RELATEDNESS OF HUMAN INTERLEUKIN-1β CONVERTASE GENE TO A *C. elegans* CELL DEATH GENE, INHIBITORY PORTIONS OF THESE GENES AND USES THEREFOR

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Related Application

This application is a continuation of USSN 08/394,189, filed February 24, 1995, which is a continuation-in-part of USSN 08/282,211, filed July 11, 1994; which is a divisional of 07/984,182, filed November 20, 1992, now abandoned; which is a continuation-in-part of USSN 07/897,788, filed June 12, 1992, now abandoned. The teachings of USSN 07/897,788 are incorporated by reference.

Background

Cell death is a fundamental aspect of animal development. Many cells die during the normal development of both vertebrates (Glucksmann, *Biol. Rev. Cambridge Philos. Soc. 26*:59-86 (1951)) and invertebrates (Truman, *Ann. Rev. Neurosci. 7*:171-188 (1984)). These deaths appear to function in morphogenesis, metamorphosis and tissue homeostasis, as well as in the generation of neuronal specificity and sexual dimorphism (reviewed by Ellis *et al., Ann. Rev. Cell Biol. 7*:663-698 (1991)). An understanding of the mechanisms that cause cells to die

and that specify which cells are to live and which cells are to die is essential for an understanding of animal development.

The nematode *Caenorhabditis elegans* is an appropriate organism for analyzing naturally-occurring or programmed cell death (Horvitz *et al., Neurosci. Comment. 1:56-65 (1982)*). The generation of the 959 somatic cells of the adult *C. elegans* hermaphrodite is accompanied by the generation and subsequent deaths of an additional 131 cells (Sulston and Horvitz, *Dev. Biol. 82*:110-156 (1977); Sulston *et al., Dev. Biol. 100*:64-119 (1982)). The morphology of cells undergoing programmed cell death in *C. elegans* has been described at both the light and electron microscopic levels (Sulston and Horvitz, *Dev. Biol. 82*:100-156 (1977); Robertson and Thomson, *J. Embryol. Exp. Morph. 67*:89-100 (1982)).

Many genes that affect *C. elegans* programmed cell death have been identified (reviewed by Ellis *et al., Ann. Rev. Cell Biol. 7*:663-698 (1991)). The activities of two of these genes, *ced-3* and *ced-4*, are required for the onset of almost all *C. elegans* programmed cell deaths (Ellis and Horvitz, *Cell 44*:817-829 (1986)). When the activity of either *ced-3* or *ced-4* is eliminated, cells that would normally die instead survive and can differentiate into recognizable cell types and even function (Ellis and Horvitz, *Cell 44*:817-829 (1986); Avery and Horvitz, *Cell 51*:1071-1078 (1987); White *et al., Phil. Trans. R. Soc. Lond. B. 331*:263-271 (1991)). Genetic mosaic analyses have indicated that the *ced-3* and *ced-4* genes most likely act in a cell autonomous manner within dying cells, suggesting that the products of these genes are expressed within dying cells and either are cytotoxic molecules or control the activities of cytotoxic molecules (Yuan and Horvitz, *Dev. Biol. 138*:33-41 (1990)).

Summary of the Invention

This invention is based mainly on two experimental findings and their implications: 1) that human interleukin-1β convertase (ICE), a cysteine protease with specificity for aspartate and which cleaves pro-interleukin-1β substrate in the P1 position to yield the active cytokine which is involved in the inflammatory response in humans, has considerable similarity to the protein encoded by the *C. elegans* cell death gene, *ced-3*; and 2) that fusion constructs containing amino-terminal portions of the *ced-3* gene can prevent cell death in *C. elegans*. As discovered by Applicant, the human ICE and nematode Ced-3 proteins have an overall amino acid identity of 28%. A higher degree of similarity was found in the carboxyl-terminal region, a region shown to be critical for the activities of both proteins. Furthermore, three sequences important for ICE activity, the region surrounding the active cysteine and two autocleavage sites, have been shown to be conserved in the *ced-3* gene product.

Thus, significant structural similarity has been shown between two proteins which previously were thought to be unrelated (to have dissimilar physiological roles). This finding leads to several implications, some of which are:

1) that the human ICE gene has an activity similar to that of *ced-3* in causing cell death;

2) that the Ced-3 protein is also a cysteine protease with a substrate specificity similar to that of ICE;

3) that mutations in the ICE gene corresponding to mutations in the *ced-3* gene will produce similar effects, such as inactivation and constitutive activation;

4) that the *ced-3* and ICE genes are members of a family of structurally related genes, referred to herein as the *ced-3*/ICE family, some of which are likely to be cell death genes and some of which may encode substrate-specific proteases;

5) that inhibitors of ICE, such as peptide aldehydes which contain the ICE recognition site or a substituted recognition site and the cowpox virus CrmA protein, may also be useful for inhibiting cell deaths; and

6) that inhibitors of *ced-3*, such as inhibitory portions of the gene or encoded product, may also be useful for inhibiting inflammation.

This hitherto unknown connection between a cell death protein and a protease involved in the inflammatory response provides a basis for developing novel drugs and methods for the treatment of acute and chronic inflammatory disease, of leukemias in which IL-1β is implicated, and of diseases and conditions characterized by cell deaths (such as myocardial infarction, stroke, traumatic brain injury, viral and other types of pathogenic infection, neural and muscular degenerative diseases such as ALS and spinal cord injury, aging, hair loss). In addition, drugs which increase cell deaths and which are useful for reducing the size or proliferative capacity of cell populations, such as cancerous cells, infected cells, cells which produce autoreactive antibodies, and hair follicle cells, as well as drugs which incapacitate or kill organisms, such as pests, parasites and recombinant organisms, can be developed using the *ced-3*, ICE, and other *ced-3*/ICE genes and their gene products.

This work also provides probes and methods for identifying additional members of the *ced-3*/ICE gene family. Genes related to *ced-3* and ICE are expected to exist in various organisms. Some of these may be cell death genes and/or proteases. The sequences of these related genes and their encoded products can be compared, for instance, using computer-based analysis, to determine their similarities. Structural comparisons, for example, would indicate those regions or features of the genes or encoded products which are likely to be functionally similar and important. Such information can be used to design drugs which mimic

or alter the activity of the *ced-3*, ICE, or other *ced-3*/ICE genes, and which may, thus, be useful in the various medical and agricultural applications mentioned above.

In addition, another mammalian protein, the murine NEDD-2 protein (also known as Ich-1), was also found to be similar to Ced-3. Interestingly, NEDD-2 is not significantly similar to ICE. Thus, another mammalian cell death gene was identified.

Also described herein is the discovery that fusion constructs which encode an amino-terminal portion of the Ced-3 protein fused to β-galactosidase act as inhibitors of cell death in *C. elegans*. Due to its structural similarity to Ced-3, constructs encoding corresponding portions of the human ICE protein are also expected to inhibit the enzymatic activity of ICE in cleaving interleukin-1β. Thus, inhibitors comprising an amino-terminal portion of the Ced-3 protein, ICE protein or another member of the Ced-3/ICE family and RNAs and DNA constructs which express these protein portions are potentially useful for decreasing cell deaths and/or inflammation involved in various pathologies. Methods for identifying other inhibitory portions of the *ced-3* and ICE genes are also described.

Furthermore, deletion of the inhibitory amino-terminal portions of the *ced-3* and ICE genes may result in constitutive activation of the genes. Constitutively activated carboxyl-terminal portions of the genes, or their encoded products, may thus be useful in applications where increased cell deaths or an increased inflammatory response are desired.

Also provided are compounds with mutations of the active site cysteine in ICE, Ced-3, CPP-32, or NEDD-2 and methods for inhibition of cell death by administering these compounds. In ICE, the active site cysteine is at position 285, in Ced-3 the active site cysteine is at position 358, in CPP-32 the active site cystein

is at position 163, and in NEDD-2 the active cysteine is at position 303. Preferably, the mutation is a missense mutation which changes one or more amino acids, including the active site cysteine. More preferably, the cysteine is replaced by an alanine or a serine. Most preferably, the cysteine is replaced by an alanine.

In another aspect, the invention features a drug for inhibiting the activity of a gene selected from the group consisting of *ced-3* and a gene which belongs to the *ced-3*/ICE gene family, comprising an inhibitor of interleukin-1β convertase. Preferably, the drug reduces cell deaths, or is a peptide aldehyde containing the amino acid sequence Tyr-Val-Xaa-Asp (SEQ ID NO: 15), wherein Xaa is selected from Ala, His, Gln, Lys, Phe, Cha, and Asp; or is Ac-Tyr-Val-Ala-Asp-CHO (SEQ ID NO: 16), also referred to as inhibitor B, or is the cowpox virus CrmA protein or a portion thereof. Preferably, the activity being inhibited is polypeptide ICE activity.

In a related aspect, the invention provides methods for inhibiting cell death by administering inhibitors of ICE or related proteases. Preferably, the peptide is a peptide aldehyde containing the amino acid sequence Tyr-Val-Xaa-Asp (SEQ ID No: 15), wherein Xaa is selected from Ala, His, Gln, Lys, Phe, Cha, and Asp; or is Ac-Tyr-Val-Ala-Asp-CHO (SEQ ID NO: 16), also referred to as inhibitor B, or is the cowpox virus CrmA protein or a portion thereof, or any protease inhibitor containing an aspartate residue in the position corresponding to the P1 site in the substrate linked to a protease-inactivating chemical moiety. Preferably, the cell death being inhibited is cell death in human nerve celles, including motoneurons. For example, the methods of the invention may be used to prevent or decrease the number of cell deaths due to amyotrophic lateral sclerosis, spinal cord injury, stroke, brain trauma, Parkinsonism, Huntington's disease,

Alzheimer's disease, or spinocerebellar degeneration (e.g., cerebello-olivary degeneration of Holmes, Friedreich's ataxia).

In another aspect, the invention features an inhibitor of the activity of the *ced-3* gene, which includes a portion of the ced-3 gene sequence. Preferably, the gene portion is a portion of the nucleotide sequence of (SEQ ID NO: 1), selected from the group consisting of:

a) nucleotides 1 to approximately 5850;

b) nucleotides 1 to approximately 3020; and

c) an inhibitory subportion (a) and (b); the gene portion encodes an amino acid sequence of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 2), selected from the group consisting of:

a) amino acids 1 to approximately 372;

b) amino acids 1 to approximately 149; and

c) an inhibitory subportion of (a) and (b).

The inhibitor of the *ced-3* gene may further include a heterologous structural gene fused 3' of the gene portion, e.g., *E. coli lacZ*, or a transcriptional signal and a translational signal suitable for expression of the gene portion in a host cell. Preferably, the transcriptional and the translational signals are those of the *ced-3* gene. In related aspects, the invention features inhibitors of the activity of the *ced-3* gene, which include RNA encoded by the sense strand of a nucleotide sequence of Fig. 3 (SEQ ID NO: 1), the nucleotide sequence being selected from the group consisting of:

a) nucleotides 1 to approximately 5850;

b) nucleotides 1 to approximately 3020; and

c) an inhibitory subportion of (a) and (b);

or an inhibitor which is a protein having an amino acid sequence of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 2), selected from the group consisting of:

a) amino acids 1 to approximately 372;

b) amino acids 1 to approximately 149; and

c) an inhibitory subportion of (a) and (b); or

which is a non-peptide mimetic of the inhibitor of the foregoing, sequences from Fig. 6A; or a construct selected from BGAFQ and PBA; or the encoded product of a construct selected from BGAFQ and PBA; or a non-peptide mimetic of the protein encoded by a construct selected from BGAFQ and PBA.

In another related aspect, the invention also features an inhibitor of the activity of the *ced-3* gene, comprising protein having an amino acid sequence of ICE shown in Fig. 6A (SEQ ID NO: 4), selected from the group consisting of:

a) amino acids 1 to 298;

b) amino acids 1 to 111; and

c) an inhibitory subportion of (a) and (b); or

which is a portion of the ICE gene which encodes the ICE, or an inhibitory subportion of said gene; or RNA encoded by the gene portion which encodes ICE; or a non-peptide mimetic of the protein of ICE. In another related aspect, the invention also features an inhibitor of the activity of the *ced-3* gene, which includes a portion of the protein product of a gene which is structurally related to the *ced-3* gene, and which protein product corresponds to an amino acid sequence of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 2), selected from the group consisting of:

a) amino acids 1 to approximately 372;

b) amino acids 1 to approximately 149; and

c) an inhibitory subportion of (a) and (b); or

an inhibitor which is a portion of a gene which is structurally related to the *ced-3* gene, and encodes one of the foregoing, *ced-3*-related amino acid fragments, or an inhibitory subsection of said gene portion; or RNA encoded by the immediately foregoing, gene portion; or a non-peptide mimetic of the foregoing, amino acid fragments which are related to *ced-3*.

In another aspect, the invention features a method for identifying a portion of the *ced-3* gene which inhibits the activity of the *ced-3* gene, which method includes the steps of:

a) injecting wild-type nematodes with a portion of the *ced-3* gene under conditions suitable for expression of said gene portion; and

b) detecting a decrease in programmed cell deaths,

whereby a decrease in programmed cell deaths is indicative of a portion of the *ced-3* gene which inhibits the activity of said gene.

In related aspects, the invention features a method of identifying a portion of a gene which is structurally related to *ced-3* and which inhibits the activity of the *ced-3* gene, wherein the structurally related DNA is substituted for the *ced-3* DNA in the immediately foregoing method. Preferably, the structurally related DNA is ICE-encoding DNA. The invention also includes isolated DNA which is identified by these methods.

In another aspect, the invention features an inhibitor of the activity of the ICE gene which includes a portion of the gene which encodes an amino sequence of ICE shown in Fig. 6A (SEQ ID NO: 4), selected from the group consisting of:

a) amino acids 1 to approximately 298;

b) amino acids 1 to approximately 111; and

c) an inhibitory subportion of (a) and (b).

This inhibitor may further include a heterologous structural gene fused 3' of the gene portion, or a transcriptional signal and a translational signal suitable for expression of the gene portion in a host cell.

In related aspects, the invention features inhibitors of the activity of the ICE gene, which include RNA encoded by the gene which encodes ICE; and inhibitors which are amino acid sequences of ICE shown in Fig. 6A (SEQ ID NO: 4), selected from the group consisting of:

a) amino acids 1 to approximately 298;

b) amino acids 1 to approximately 111;

c) an inhibitory subportion of (a) and (b);

which is a non-peptide mimetic of the immediately foregoing, amino acid fragments; and a portion of the *ced-3* gene. Preferably, the inhibitory portion of the *ced-3* gene is a nucleotide sequence of Fig. 3 (SEQ ID NO: 1), selected from the group consisting of:

a) nucleotides 1 to approximately 5850;

b) nucleotides 1 to approximately 3020;

c) an inhibitory subportion of (a) and (b); or

is a nucleotide sequence which encodes an amino acid sequence of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 2), selected from the group consisting of:

a) amino acids 1 to approximately 372;

b) amino acids 1 to approximately 149; and

c) an inhibitory subportion of (a) and (b); or

is an inhibitor which is a nucleotide sequence including a construct selected from BGAFQ and PBA, or which is the encoded products thereof. In one embodiment, the nucleic acid inhibitor further includes a heterologous structural gene fused 3' of

the gene portion, or a transcriptional signal and a translational signal suitable for expression of the gene portion in a host cell.

In related aspects, the invention features inhibitors of the activity of the ICE gene, including RNA encoded by the sense strand of a portion of the *ced-3* gene, which is a nucleotide sequence of Fig. 3 (SEQ ID NO: 1), selected from the group consisting of:

a) nucleotides 1 to approximately 5850;

b) nucleotides 1 to approximately 3020; and

c) an inhibitory subportion of (a) and (b);

and an inhibitor which is a protein having an amino acid sequence of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 4), selected from the group consisting of:

a) amino acids 1 to approximately 372;

b) amino acids 1 to approximately 149; and

c) an inhibitory subportion of (a) and (b); or

an inhibitor which is a protein having an amino acid sequence of the *ced-3* protein shown in Fig. 6A (SEQ ID NO: 4), selected from the group consisting of:

a) amino acids 1 to approximately 372;

b) amino acids 1 to approximately 149; and

c) an inhibitory subportion of (a) and (b); or

an inhibitor which is a non-peptide mimetic of the immediately foregoing, protein fragments.

In a further related aspect, the invention features an inhibitor of the activity of the ICE gene which includes a portion of the protein product of a gene which is structurally related to said ICE gene, which portion corresponds to an amino acid sequence of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 2), selected from the group consisting of:

a) amino acids 1 to approximately 372;

b) amino acids 1 to approximately 149; and

c) an inhibitory subportion of (a) and (b); or

an inhibitor which is a portion of a gene which is structurally related to the ICE gene, which gene encodes one of the immediately foregoing, amino acid sequences, or an inhibitory subsection of such a gene which is structurally related to a gene encoding the foregoing, protein fragments; or RNA encoded by the gene which encodes the foregoing, protein fragments; or a non-peptide mimetic of the foregoing, protein fragments.

In another aspect, the invention features a method for identifying a portion of ICE which inhibits the activity of said ICE, comprising the steps of:

a) combining a portion of ICE with ICE and a substrate of ICE under conditions suitable for cleavage of the substrate by ICE; and

b) detecting a decrease in cleavage of the substrate, whereby a decrease in cleavage of the substrate is indicative of a portion of ICE which inhibits the activity of said enzyme.

In a related aspect, the invention features an isolated inhibitory portion of the ICE protein identified by this method and nucleic acid encoding this inhibitory portion.

In another aspect, the invention features a method for identifying a portion of the protein product of a gene which is structurally related to the *ced-3* and ICE genes, and which inhibits the activity of ICE, comprising the steps of:

a) combining a portion of the protein product of a gene which is structurally related to the *ced-3* and ICE genes with ICE and a

substrate of ICE under conditions suitable for cleavage of the substrate by ICE; and

b) detecting a decrease in cleavage of the substrate,

whereby a decrease in cleavage of the substrate is indicative of a portion of the protein product of a gene which is structurally related to the *ced-3* and ICE genes and inhibits the activity of ICE. In related aspects, the invention features an isolated inhibitory portion identified by the method and isolated nucleic acid encoding the inhibitory portion identified by the method.

In other aspects, the invention features inhibitors of the activity of a gene belonging to the *ced-3*/ICE family of structurally related genes, comprising DNA selected from the group consisting of:

a) a portion of the nucleotide sequence of Fig. 3 (SEQ ID NO: 1), selected from the group consisting of:

1) nucleotides 1 to approximately 5850;

2) nucleotides 1 to approximately 3020; and

3) an inhibitory subportion of (a.1) and (a.2);

b) DNA encoding an amino acid sequence of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 2), selected from the group consisting of:

1) amino acids 1 to approximately 372;

2) amino acids 1 to approximately 149; and

3) an inhibitory subportion of (b.1) and (b.2);

c) a portion of the ICE gene which encodes an amino acid sequence of ICE shown in Fig. 6A (SEQ ID NO: 4), selected from the group consisting of:

1) amino acids 1 to approximately 298;

2) amino acids 1 to approximately 111; and

3) an inhibitory subportion of (c.1) and c.2);

d) a portion of the *ced-3*/ICE gene which encodes an amino acid sequence corresponding to a portion of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 2), which Ced-3 portion selected from the group consisting of:

1) amino acids 1 to approximately 372;

2) amino acids 1 to approximately 149; and

3) an inhibitory subportion of (d.1) and (d.2); and

e) a portion of a *ced-3*/ICE gene other than the *ced-3*/ICE gene which encodes an amino acid sequence corresponding to a portion of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 2), said Ced-3 portion selected from the group consisting of:

1) amino acids 1 to approximately 372;

2) amino acids 1 to approximately 149; and

3) an inhibitory subportion of (e.1) and (e.2); or comprising RNA encoded by the DNA of a) - e), immediately above; or protein encoded by the DNA of a) - e), immediately above; or a non-peptide mimetic of the proteins and fragments encoded by the DNA of a) - e), immediately above.

In another aspect, the invention features a drug for reducing cell deaths, which includes an inhibitor of the activity of the *ced-3* gene, selected from the group consisting of:

a) a portion of the *ced-3* gene;

b) a product encoded by a portion of the *ced-3* gene;

c) a non-peptide mimetic of an inhibitory portion of the Ced-3 protein;

d) a portion of the ICE gene;

e) a product encoded by a portion of the ICE gene;

f) a non-peptide mimetic of an inhibitory portion of the ICE protein;

g) a portion of a gene which is structurally related to the *ced-3* gene;

h) a product encoded by the gene portion of (g); and

i) a non-peptide mimetic of the protein encoded by the gene portion of (g).

Preferably, the inhibitor is selected from the group consisting of:

a) DNA having a nucleotide sequence of Fig. 3 (SEQ ID NO: 1), selected from the group consisting of:

1) nucleotides 1 to approximately 5850;

2) nucleotides 1 to approximately 3020; and

3) an inhibitory portion of (a.1) and (a.2);

b) DNA encoding an amino acid sequence of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 2), selected from:

1) amino acids 1 to approximately 372;

2) amino acids 1 to approximately 149; and

3) an inhibitory portion of (b.1) and (b.2);

c) RNA encoded by DNA of (a);

d) RNA encoded by DNA of (b);

e) protein having an amino acid sequence of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 2), selected from the group consisting of:

1) amino acids 1 to approximately 372;

2) amino acids 1 to approximately 149; and

3) inhibitory portion of (e.1) and (e.2); and

f) a non-peptide mimetic of the protein of e); or selected from the group consisting of:

g) DNA encoding an amino acid sequence of ICE shown in Fig. 6A (SEQ ID NO: 4), selected from the group consisting of:

1) amino acids 1 to approximately 298;

2) amino acids 1 to approximately 111; and

3) an inhibitory portion of (g.1) and (g.2);

h) RNA encoded by DNA of g);

i) protein having an amino acid sequence of ICE shown in Fig. 6A (SEQ ID NO: 4), selected from the group consisting of:

1) amino acids 1 to approximately 298;

2) amino acids 1 to approximately 111; and

3) an inhibitory portion of (i.1) and (i.2); and

j) a non-peptide mimetic of the protein of i); or selected from the group consisting of:

k) protein encoded by a portion of a gene which is structurally related to the *ced-3* gene, said protein portion corresponding to an amino acid sequence of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 2) selected from the group consisting of:

1) amino acids 1 to approximately 372;

2) amino acids 1 to approximately 149; and

3) an inhibitory portion of (k.1) and (k.2);

l) DNA encoding the protein of (k) or inhibitory subportion thereof;

m) RNA encoding the protein of (k) or inhibitory subportion thereof; and

n) a non-peptide mimetic of the protein of (k).

In a related aspect, the invention features a method for treating a condition characterized by cell deaths, comprising administering the drug of which is an inhibitor of the activity of the *ced-3* gene or protein.

In another aspect, the invention features a drug for reducing cell deaths, which includes an inhibitor of the activity of the ICE gene or protein, selected from the group consisting of:

a) a portion of the ICE gene;

b) a product encoded by a portion of the ICE gene;

c) a non-peptide mimetic of an inhibitory portion of the ICE protein;

d) a portion of the *ced-3* gene;

e) a product encoded by a portion of the *ced-3* gene;

f) a non-peptide mimetic of an inhibitory portion of the Ced-3 protein;

g) a portion of a gene which is structurally related to the *ced-3* gene and the ICE gene;

h) a product encoded by the gene portion of (e); and

i) a non-peptide mimetic of the protein encoded by (g).

Preferably, the drug is structurally related to the *ced-3* gene and the ICE gene, and is selected from the group consisting of:

a) a portion of said related gene;

b) a product encoded by the gene portion of (a);

c) a non-peptide mimetic of the protein product encoded by (a);

d) a portion of the ICE gene;

e) a product encoded by the gene portion of (d);

f) a non-peptide mimetic of a protein product encoded by (d);

g) a portion of the *ced-3* gene;

h) a product encoded by the gene portion of (g); and

i) a non-peptide mimetic of the protein product encoded by (g).

In another aspect, the invention features an anti-inflammatory drug, comprising an inhibitor of the activity of the ICE gene or protein, or inhibitory portion thereof, selected from the group consisting of:

a) a portion of the ICE gene;

b) a product encoded by a portion of the ICE gene;

c) a portion of the *ced-3* gene;

d) a product encoded by a portion of the *ced-3* gene;

e) a portion of a gene which is structurally related to the *ced-3* gene and ICE gene; and

f) a product encoded by a portion of a gene which is structurally related to the *ced-3* gene and the ICE gene.

Preferably, the anti-inflammatory drug is an inhibitor selected from the group consisting of:

a) DNA encoding an amino acid sequence of ICE shown in Fig. 6A (SEQ ID NO: 4), selected from the group consisting of:

1) amino acids 1 to approximately 298;

2) amino acids 1 to approximately 111; and

3) an inhibitory portion of (a.1) and (a.2);

b) RNA encoded by DNA of (a) or an inhibitory subportion thereof;

c) protein having an amino acid sequence of ICE shown in Fig. 6A (SEQ ID NO: 4), selected from the group consisting of:

1) amino acids 1 to approximately 298;

2) amino acids 1 to approximately 111; and

3) an inhibitory portion of (c.1) and (c.2);

d) a non-peptide mimetic of the protein of (c); or the inhibitor is selected from the group consisting of:

e) DNA having a nucleotide sequence of Fig. 3 (SEQ ID NO: 1), selected from the group consisting of:

1) nucleotides 1 to approximately 5850;

2) nucleotides 1 to approximately 3020; and

3) an inhibitory portion of (e.1) and (e.2);

f) DNA encoding an amino acid sequence of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 2), selected from the group consisting of:

1) amino acids 1 to approximately 372;

2) amino acids 1 to approximately 149; and

3) an inhibitory portion of (f.1) and (f.2);

g) RNA encoded by DNA of (e);

h) RNA encoded by DNA of (f);

i) protein having an amino acid sequence of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 2), selected from the group consisting of:

1) amino acids 1 to approximately 372;

2) amino acids 1 to approximately 149; and

3) an inhibitory portion of (i.1) and (i.2); and

k) a non-peptide mimetic of the protein of (i); or the inhibitor is selected from the group consisting of:

l) protein encoded by a portion of a gene which is structurally related to the *ced-3* and ICE genes, said protein portion corresponding to an amino acid sequence of ICE shown in Fig. 6A (SEQ ID NO: 4), selected from the group consisting of:

1) amino acids 1 to approximately 298;

2) amino acids 1 to approximately 111; and

3) an inhibitory portion of (l.1) and (l.2);

m) DNA encoding the protein of (l);

n) RNA encoding the protein of (l); and

o) a non-peptide mimetic of the protein of (l).

In related aspects, the invention features methods for treating inflammation, which includes administering the drug of a) - o), immediately above.

In another aspect, the invention features a method for altering the occurrence of cell death, which includes altering the activity of a cell death gene which is structurally related to *ced-3*. Preferably, the structurally related gene is ICE.

In another aspect, the invention features a drug for increasing cell deaths, which includes a molecule, or active portion thereof, selected from:

a) DNA comprising a gene which belongs to the *ced-3*/ICE gene family;

b) RNA encoded by the DNA of (a);

c) protein encoded by the DNA of (a);

d) an agent which is structurally similar to and mimics the activity of the protein of (c);

e) an agonist of the activity of a gene which belongs to the *ced-3*/ICE gene family;

f) DNA comprising a constitutively activated mutated form of a gene which belongs to the *ced-3*/ICE gene family;

g) RNA encoded by the DNA of (e);

h) protein encoded by the DNA of (e);

i) an agent which is structurally similar to and mimics the activity of a protein encoded by the DNA of (e); and

j) an agonist of the activity of a constitutively activated mutated form of a gene which belongs to the *ced-3*/ICE gene family.

In a related aspect, the invention features the drug of a) - f), immediately foregoing, wherein the gene which belongs to the *ced-3*/ICE gene family is ICE. Preferably, where drug is a constitutively activated mutated form of the gene which belongs to the *ced-3*/ICE gene family encodes a carboxyl-terminal portion of a protein product of the wild-type gene, the carboxyl-terminal portion having a deletion of an amino-terminal portion which corresponds to an amino acid sequence of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 2), selected from the group consisting of:

k) amino acids 1 to approximately 372;

l) amino acids 1 to approximately 149; and

m) an inhibitory subportion of (h) and (l).

More preferably, the protein product of the wild-type gene has sequences corresponding to the autocleavage sites of ICE and the protein product of the wild-type gene is selected from the group consisting of:

n) the uncleaved form of the protein product; and

o) the subunits corresponding to the active subunits of ICE.

In a related aspect, the invention features a method for reducing the proliferative capacity or size of a population of cells, including contacting the cells with the drug for increasing cell deaths selected from the immediately foregoing, group a) - j), under conditions suitable for activity of the drug. Preferably, the population of cells is selected from the group consisting of:

a) cancerous cells;

b) cells which produce autoreactive antibodies;

c) infected cells;

d) hair follicle cells;

e) cells which are critical to the life of a parasite;

f) cells which are critical to the life of a pest; and

g) cells which are critical to the life of a recombinant organism.

In another aspect, the invention features a drug for decreasing cell deaths comprising a molecule selected from the group consisting of:

a) single stranded nucleic acid having all or a portion of the antisense sequence of a gene which is structurally related to *ced-3*, said nucleic acid which is complementary to the mRNA of the gene;

b) DNA which directs the expression of (a);

c) a mutated form of a gene which is structurally related to *ced-3*, does not cause cell death and antagonizes the activity of the wild-type gene; and

d) an antagonist of the activity of a gene which is structurally related to *ced-3*.

Preferably, the structurally related gene is ICE.

In a related aspect, the invention features a method for treating, in a human or other animal, a condition characterized by cell deaths, which method includes administering the drug of a) - d), immediately foregoing, to the human or other animal under conditions suitable for activity of the drug. Preferably, the condition is selected from the group consisting of:

a) myocardial infarction;

b) stroke;

c) degenerative disease;

d) traumatic brain injury;

e) hypoxia;

f) pathogenic infection; and

g) hair loss.

In another aspect, the invention features a diagnostic probe for a disease characterized by cell deaths, comprising a molecule selected from the group consisting of:

a) all or a portion of the *ced-3* gene (SEQ ID NO: 1) which is specific to said *ced-3* gene;

b) RNA encoded by the *ced-3* gene;

c) degenerate oligonucleotides derived from the amino acid sequence of the Ced-3 protein (SEQ ID NO: 2);

d) an antibody directed against the Ced-3 protein;

e) all or a portion of the ICE gene (SEQ ID NO: 3) which is specific to said ICE gene;

f) RNA encoded by the ICE gene;

g) degenerate oligonucleotides derived from the amino acid sequence of ICE (SEQ ID NO: 4);

h) an antibody directed against ICE;

i) a gene which is structurally related to the *ced-3* gene, or portion thereof specific to said structurally related gene;

j) RNA encoded by the structurally related gene;

k) degenerate oligonucleotides derived from the amino acid sequence of the protein product of a gene which is structurally related to *ced-3*; and

d) an antibody directed against the protein product of a gene which is structurally related to *ced-3*.

In related aspects, the invention provides methods for diagnosis of a diseases characterized by cell deaths, which included detecting an abnormality in the sequence of a gene which is structurally related to *ced-3*; or which includes detecting an abnormality in the activity of a gene which is structurally related to *ced-3*. Preferably, the structurally related gene is ICE. In another aspect, the invention provides a diagnostic probe for an inflammatory disease, which includes a molecule selected from the group consisting of:

a) all or a portion of the *ced-3* gene shown in Fig. 3 (SEQ ID NO: 1) which is specific to the *ced-3* gene;

b) RNA encoded by (a);

c) degenerate oligonucleotides derived from the amino acid sequence of the Ced-3 protein as shown in Fig. 6A (SEQ ID NO: 2);

d) an antibody directed against the Ced-3 protein;

e) a gene which is structurally related to the *ced-3* and ICE genes, or portion thereof which is specific for said related gene;

f) RNA encoded by (a);

g) degenerate oligonucleotides derived from the amino acid sequence of the protein encoded by (e); and

h) an antibody directed against the protein encoded by (e).

In a related aspect, the invention features a method for diagnosis of an inflammatory disease, which includes detecting an abnormality in the sequence of a gene which is a member of the *ced-3*/ICE gene family; or which includes detecting an abnormality in the activity of a gene which belongs to the *ced-3*/ICE gene family, or an encoded product thereof. Preferably, the gene which is a member of the *ced-3*/ICE family is *ced-3*.

In another aspect, the invention features an isolated substrate-specific protease having the amino acid sequence of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 2). In a related aspect, the invention provides an isolated substrate-specific protease, consisting essentially of a protein product of a gene which is structurally related to the *ced-3* and ICE genes. Preferably, the protease cleaves after aspartate residues or is a cysteine protease.

In another aspect, the invention features isolated ICE having an alteration which reduces the activity of the enzyme, the alteration selected from the group consisting of:

a) Lysine to Phe at amino acid 26;

b) Gly to Arg at amino acid 65;

c) Cys to Ala or Ser at amino acid 285;

d) Gly to Ser at amino acid 287;

e) Glu to termination at amino acid 324;

f) Trp to termination at amino acid 340;

g) Ala to Val at amino acid 361;

h) Glu to Lys at amino acid 390; and

i) Thr to Phe at amino acid 393.

The invention also provides methods for inhibiting cell death by administering the ICE polypeptides of a)-i), above.

In related aspects, the invention provides isolated DNA which encodes a mutated ICE having the amino acid alterations specified in a) - h), immediately foregoing, and RNA encoded by this DNA.

In another aspect, the invention features an isolated gene belonging to the *ced-3*/ICE family of structurally related genes which has a mutation conferring reduced activity of the gene, said mutation resulting in an amino acid alteration corresponding to an amino acid alteration of the Ced-3 protein which inactivates the Ced-3 protein. The product of the gene may be either RNA or protein.

In another aspect, the invention features a constitutively activated cell death protein comprising an amino acid sequence of the Ced-3 protein shown in Fig. 6A (SEQ ID NO: 2), selected from the group consisting of:

a) the amino acids from approximately 150 to 503;

b) the amino acids from approximately 373 to 503;

c) the amino acids from approximately 150 to 372;

d) (b) and (c) together;

e) an active subportion of (a), (b), and (c); and

f) combinations of a) - e).

Preferably, the constitutively activated protein further includes a subportion of the region of Ced-3 from amino acids 1 to 149, as shown in Fig. 6A (SEQ ID NO: 2), which subportion which enhances and does not inhibit the activity of the protein.

In related aspects, the invention features drugs for increasing cell deaths, including a molecule selected from the proteins of a) - f), immediately foregoing, or a nucleic acid encoding said protein. In a related aspect, the invention features isolated nucleic acid encoding the proteins a) - f), immediately foregoing.

In another aspect, the invention features constitutively activated cell death protein having an amino acid sequence of ICE shown in Fig. 6A (SEQ ID NO: 4), selected from the group consisting of:

a) the amino acids from approximately 111 to 404;

b) the amino acids from approximately 298 to 404;

c) the amino acids from approximately 111 to 297;

d) (b) and (c) together;

e) an active subportion of (a), (b), and (c); and

f) combinations of these.

In a related aspect, the invention features isolated nucleic acid encoding a protein of a) - f), immediately foregoing.

In another aspect, the invention features a method for identifying a gene which is structurally related to the *ced-3* gene and the ICE gene, comprising detecting a gene with:

a) a probe derived from the *ced-3* gene or a product encoded by the *ced-3* gene; and

b) a probe derived from the ICE gene or a product encoded by the ICE gene, and

a method for identifying a gene which belongs to the *ced-3*/ICE family of structurally related genes, comprising detecting a gene with a probe selected from the group consisting of:

a) a probe derived from a gene which is structurally related to the *ced-3* gene and the ICE gene; and

b) a probe derived from the consensus sequence of a conserved region in genes belonging to the *ced-3*/ICE gene family.

In related aspects, the invention provides isolated genes identified by these methods. Preferably, the isolated gene has a cell death activity, a protease activity, or both.

In another aspect, the invention provides isolated DNA selected from the group consisting of:

a) a region of a gene belonging to the *ced-3*/ICE family of structurally related genes which is conserved among two or more family members; and

b) the consensus sequence of a conserved region in genes belonging to the *ced-3*/ICE gene family,

or encoded product thereof.

In another aspect, the invention provides a method for identifying a gene which interacts with a *ced-3*/ICE gene belonging to this family, which includes identifying a mutation which enhances or suppresses the activity of a *ced-3*/ICE gene in a nematode, whereby the enhancing or suppressing mutation is indicative of a gene which interacts with the *ced-3*/ICE gene. Preferably, the *ced-3*/ICE gene is selected from the group consisting of:

a) a wild-type *ced-3* gene;

b) a mutated *ced-3* gene, the nematode being a mutant nematode;

c) a transgene which is a wild-type form of said *ced-3*/ICE gene, the nematode being a transgenic nematode having an inactivated endogenous *ced-3* gene; and

d) a transgene which is a mutated form of said *ced-3*/ICE gene, the nematode being a transgenic nematode having an inactivated endogenous *ced-3* gene. In a related aspect, the invention provides an isolated gene identified by the above method.

In another aspect, the invention provides a bioassay for identifying an agent which affects the activity of a gene belonging to the *ced-3*/ICE family of structurally related genes, comprising the steps of:

a) introducing an agent into a transgenic nematode which expresses a *ced-3*/ICE gene; and

b) detecting an alteration in the occurrence of cell deaths in the transgenic nematode, wherein an alteration indicates that the agent affects the activity of the *ced-3*/ICE gene.

Preferably, the *ced-3*/ICE gene is selected from a wild-type gene and a mutated gene. In a related aspect, the invention features an agent identified by the bioassay.

In another aspect, the invention features an isolated protein having cell death activity and the amino acid sequence of the NEDD-2 protein shown in Fig. 6B (SEQ ID NO: 13), or an active portion thereof and isolated nucleic acid encoding the protein. In a related aspect the invention features isolated NEDD-2 protein having an alteration which inactivates the protein, said alteration selected from the group consisting of:

a) Ala to Val at amino acid 117;

b) Cys to Ser or Ala at amino acid 303;

c) Glu to Lys at amino acid 483; and

d) Ser to Phe at amino acid 486; and

isolated nucleic acid encoding the protein.

In another aspect, the invention features isolated protein which is structurally similar to Ced-3 and has an alteration at a conserved amino acid corresponding to an amino acid of the Ced-3 protein selected from the group consisting of:

a) Ser 183;

b) Met 234;

c) Arg 242;

d) Leu 246;

e) Ile 247;

f) Ile 248;

g) Asn 250;

h) Phe 253;

i) Arg 259;

j) Gly 261;

k) Asp 265;

l) Gly 277;

m) Tyr 278;

n) Val 280;

o) Lys 283;

p) Asn 285;

q) Leu 286;

r) Thr 287;

s) Met 291;

t) Phe 298;

u) His 304;

v) Asp 306;

w) Ser 307;

x) Leu 310;

y) Val 311;

z) Ser 314;

aa) His 315;

bb) Gly 316;

cc) Ile 321;

dd) Gly 323;

ee) Ile 334;

ff) Asn 339;

gg) Pro 344;

hh) Leu 346;

ii) Lys 349;

jj) Pro 350;

kk) Lys 351;

ll) Gln 356;

mm) Ala 357;

nn) Cys 358;

oo) Arg 359;

pp) Gly 360;

qq) Asp 371;

rr) Asp 414;

ss) Arg 429;

tt) Gly 434;

uu) Ser 435;

vv) Ile 438;

ww) Ala 449;

xx) Val 452;

yy) Leu 488;

aa) Tyr 493;

aaa) Pro 496; and

isolated nucleic acid encoding these proteins.

By asp-ase is meant a protease which specifically cleaves a substrate after aspartate residues and therefore has a requirement for an aspartate in the P1 position of the substrate pocket. For example, ICE, granzyme B, prICE, NEDD-2, CPP-32 (Fernandes-Alnemri et al., *J. Biol. Chem.* 269:30761 (1994)), ICE-2, and ICE-4 are all asp-ases. Preferably, the asp-ase is ICE.

By inhibitors of asp-ases is meant any compound which decreases the enzymatic activity of an asp-ase by more than 5%, more preferably by more than 25%, and most preferably by more than 60% under standard *in vitro* assay condition. See, for example, Thornberry et al. (Thornberry *et al.*, *Nature 356*:768-774 (1992)) and Lazednik et al. (Lazednik et al., *Nature* 371:346-347 (1994)) for appropriate assay conditions. Examples of several asp-ase inhibitors are provided herein.

Brief Description of the Drawings

Fig. 1 shows the physical and genetic maps of the *ced-3* region on chromosome IV.

Fig. 2 summarizes the experiments to localize *ced-3* within C48D1. Restriction sites of plasmid C48D1 and subclone plasmids are shown. Ced-3 activity was scored as the number of cell corpses in the head of L1 young animals.

++, the number of cell corpses above 10. +, the number of cell corpses below 10 but above 2. -, the number of cell corpses below 2.

Fig. 3 shows the nucleotide sequence (SEQ ID NO: 1) of *ced-3* and deduced amino acid sequence (SEQ ID NO: 2). The genomic sequence of the *ced-3* region was obtained from plasmid pJ107. The introns and the positions of 12 *ced-3* mutations are indicated. The likely translation initiation site is indicated by a solid arrowhead. The SL1 splice acceptor of the RNA is boxed. Repetitive elements are indicated as arrows above the relevant sequences. Numbers on the sides indicate nucleotide positions. Numbers under the amino acid sequence indicate codon positions.

Fig. 4A shows the genomic structure of the *ced-3* gene and the location of the mutations. The sizes of the introns and exons are given in bp. The downward arrows indicate the positions of 12 EMS-induced mutations of *ced-3*. The arrow pointing right indicates the direction of transcription. The solid arrowhead indicates the translation initiation site. The open arrowhead indicates the termination codon.

Fig. 4B shows the locations of the mutations relative to the exons (numbered 1-7) and the encoded serine-rich region in *ced-3*.

Fig. 5 shows a Kyte-Doolittle hydrophobicity plot of the Ced-3 protein.

Fig. 6A shows the alignment of the amino acid sequences of Ced-3 (SEQ ID NO: 2) and human interleukin-1β convertase (ICE; SEQ ID NO: 4). Vertical bars indicate identical amino acids and single and double dots indicate similar amino acids, where double dots signifies closer similarity than a single dot. The serine-rich region and inactivating mutations of Ced-3 are indicated. The active site and autocleavage sites of ICE are indicated. The portions of the Ced-3 protein encoded by the BGAFQ and PBA constructs are also shown.

Fig. 6B shows the alignment of the amino acid sequences of Ced-3 (SEQ ID NO: 2) and murine NEDD-2 (SEQ ID NO: 13). Vertical bars and single and double dots signify degrees of similarity as in Figure 6A. Inactivating mutations of Ced-3 are shown.

Fig. 6C shows the alignment of the amino-terminal regions of the Ced-3 proteins of three nematode species (*C. briggsae*, *C. elegans*, and *C. vulgaris*) and mouse (SEQ ID NO: 14) and human ICEs. A consensus sequence is also shown. Amino acid positions with the same residue in more than half of the sequences are shaded. Completely conserved amino acids are also boxed.

Fig. 6D shows the alignment of carboxyl-terminal regions of the three nematode Ced-3 proteins, human and mouse ICEs, and the nouse NEDD-2 protein. Except for NEDD-2, these sequences are contiguous with the corresponding sequences shown in Figure 6C. A consensus sequence and amino acid conservation are also shown.

Fig. 7 shows a comparison of the Ced-3 proteins of *C. elegans* (line 1; SEQ ID NO: 2) and two related nematode species, *C. briggsae* (line 2; SEQ ID NO: 5) and *C. vulgaris* (line 3; SEQ ID NO: 6). The conserved amino acids are indicated by ".". Gaps inserted in the sequence for the purpose of alignment are indicated by "_".

Fig. 8 shows the interleukin-1β convertase cDNA sequence (SEQ ID NO: 3).

Fig. 9A shows a schematic representation of two fusion constructs that can prevent programmed cell death.

Fig. 9B shows a schematic representation of the *lacZ*-containing portion of the fusion constructs.

Fig. 10 shows a schematic representation of the Cysteine 358 to Alanine construct and the decrease in all deaths conferred by the presence of this contruct in *C. elegans*.

Figs. 11A-11E show the effectiveness of peptide inhibitors of ICE in arresting motoneuron death. Fig. 11A shows the results obtained with Ac-YVAD-CHO. Fig. 11B shows the results obtained with a chloromethylketone peptide inhibitor of ICE (Ac-YVAD-CMK); Fig. 11C shows the results obtained with leupeptin, a control peptide aldehyde protease inhibitor (Ac-LLR-CHO); and Figs. 11D and 11E show the results obtained with a control chloromethylketone protease inhibitor, Tos-Lys-CMK, (Fig. 11D) or the membrane permeable calpain inhibitor Ed64 (Fig. 11E).

Figs. 12A and 12B show that the inhibition of ICE delays the death of motoneurons. Fig. 12A shows the effect of Ac-YVAD-CHO, and Fig. 12B shows the effect of Ac-YVAD-CHO. Results are expressed as % control, where control represents cultures supplied with muscle extract at plating

Fig. 13 shows that the effectiveness of the peptide aldehyde inhibitor of ICE (Ac-YVAD-CHO) for inhibition of motoneuron PCD *in vivo* is dose dependent.

Fig. 14 shows that peptide inhibitors of ICE prevent the cell death of hind limb interdigital cell death.

Fig. 15 shows photomicrographs of SCl immunopositive motoneurons cultured for 6 days with muscle extract (Panel C), without muscle extract (Panel D), with muscle extract and treated with the aldehyde peptide inhibitor of ICE(Ac-YVAD-CHO) as described in Example 4 (Panel E; arrows indicate two SCl immunopositive motoneurons), without muscle extract and treated with the peptide inhibitor of ICE (Panel F), or initially plated without muscle extract, treated with

the peptide inhibitor of ICE, then treated with muscle extract at three days in culture (Panel G). Scale bar = 25 μm.

Detailed Description of the Invention

This invention is based on the discovery that the human enzyme interleukin-1β convertase (ICE) has significant structural similarity to the protein product of the *C. elegans* cell death gene, *ced-3*. The activities of *ced-3* and another cell death gene, *ced-4*, have been shown to be required for almost all the cell deaths which occur during the development of the nematode. ICE is a cysteine protease whose physiological significance has been thought to be related to its role in the maturation of one form of interleukin-1 (IL-1), a major mediator of the immune and inflammatory response (Fuhlbrigge *et al.*, in: *The Year in Immunology*, Cruse and Lewis (eds.), Karger, Basel, 1989, pp. 21-37). There are two distantly related forms of IL-1, α and β, of which the β form is the predominant species. ICE selectively converts pro-interleukin-1β to the active cytokine, IL-1β. The production of active IL-1β has been implicated in acute and chronic inflammatory diseases, septic shock, and other physiological processes, including wound healing and resistance to viral infection (Ray *et al.*, *Cell 69*:597-604 (1992)). As a result of this discovery, an enzyme which has been known to be involved in the inflammatory response and inflammatory diseases is implicated as having a role in cell death processes. This discovery is consistent with the notion that cell death genes equivalent to the nematode *ced-3* gene function in a variety of organisms. The structural similarity between their gene products suggests that the ICE gene is a human equivalent of the *ced-3* cell death gene. As further described below, the conservation of certain features of ICE in the Ced-3 protein further suggests that Ced-3 is a protease with a substrate-specificity similar to that of ICE.

Furthermore, the identification of *ced-3* and ICE as structurally related genes (i.e., genes whose encoded products, or which themselves, are structurally similar) presents the possibility that a family of structurally related genes exists and provides probes to identify additional members of this *ced-3*/ICE gene family. Comparison of the genes within this family could indicate functionally important features of the genes or their gene products, and thus, provide information for designing drugs which are useful for treating conditions characterized by cell deaths and/or inflammatory disease.

This discovery provides novel drugs based on the *ced-3*, ICE and other *ced-3*/ICE genes and encoded products that inhibit the production of IL-1β and are useful for treatment (preventive and therapeutic) of acute and chronic inflammatory disease, as well as drugs which reduce cell deaths and are useful for treatment of diseases and conditions involving cell deaths (such as myocardial infarction, stroke, traumatic brain injury, viral and other types of pathogenic infection, degenerative diseases, aging, and hair loss). These drugs may also be useful for treating leukemias in which IL-1β has been implicated.

Drugs or agents which increase cell deaths can also be developed based on the *ced-3*, ICE, and related genes and gene products; such drugs or agents may be useful for killing or incapacitating undesired cell populations (such as cancerous cells, infected cells, cells which produce autoreactive antibodies and hair follicle cells) or undesired organisms (such as pests, parasites, and genetically engineered organisms). Drugs are also provided which increase IL-1β production and, therefore, the inflammatory and immune response. These drugs may be helpful for providing increased resistance to viral and other types of infection.

Also described herein is the discovery that fusion constructs containing amino-terminal portions of the *ced-3* gene can inhibit the activity of the intact gene

when expressed in otherwise wild-type worms. Due to the similarity between ICE and Ced-3, it is likely that the corresponding amino-terminal portions of the ICE gene will also inhibit the enzymatic activity of ICE in cleaving interleukin-1β. Thus, novel inhibitors of the *ced-3* and ICE genes are provided which may be useful for decreasing cell deaths and/or inflammation involved in various pathologies.

This work has also shown that Ced-3 and the murine NEDD-2 protein are structurally similar. Thus, drugs for increasing or decreasing cell deaths can be developed based on the NEDD-2 gene and its encoded products.

The above-described discoveries, and their implications, and novel drugs and treatments for diseases related to cell death and/or inflammation arising therefrom are described in further detail below.

As used herein, the activity of a gene is intended to include the activity of the gene itself and of the encoded products of the gene. Thus, drugs and mutations which affect the activity of a gene include those which affect the expression as well as the function of the encoded RNA and protein. The drugs may interact with the gene or with the RNA or protein encoded by the gene, or may exert their effect more indirectly.

It is understood that many of the methods used herein may be utilized in a therapeutic context. Where the therapeutic compound is DNA it is understood that method known in the art of gene therapy may be employed for therapeutic drug delivery. For example, *in vivo* or *ex vivo* methods may be used to provide DNA encoding therapeutic peptides which prevent cell death to organs and tissues used for transplantation. Similarly, such techniques may be used to administer nucleic acid to a patient suffering a cell death disease. Where peptide and peptide

mimetics are to be employed standard techniques known in the pharmaceutical art may be used to determine the most effective dosage and route of delivery.

The *ced-3* Gene

The *C. elegans ced-3* gene was cloned by mapping DNA restriction fragment length polymorphisms (RFLPs) and chromosome walking (Example 1; Figure 1). The gene was localized to a 7.5 kb fragment of cloned genomic DNA by complementation of the *ced-3* mutant phenotype (Figure 2). A 2.8 kb transcript was further identified. The *ced-3* transcript was found to be most abundant in embryos, but was also detected in larvae and young adults, suggesting that *ced-3* is expressed not only in cells undergoing programmed cell death.

A 2.5 kb cDNA corresponding to the *ced-3* mRNA was sequenced. The genomic sequence cloned in the plasmid pJ107 was also determined (Figure 3; SEQ ID NO: 1). A comparison with the cDNA sequence revealed that the *ced-3* gene has 7 introns which range in size from 54 to 1195 bp (Figure 4A). The four largest introns, as well as sequences 5' of the start codon, contain repetitive elements (Figure 3), some of which have been previously characterized in non-coding regions of other *C. elegans* genes such as *fem-1* (Spence *et al., Cell 60*:981-990 (1990)), *lin-12*, and *myoD* (Krause *et al.*, *Cell 63*:907-919 (1990)). The transcriptional start site was also mapped (Figure 3), and a *ced-3* transcript was found to be trans-spliced to a *C. elegans* splice leader, SL1.

Twelve EMS-induced *ced-3* alleles were also sequenced. Eight of the mutations are missense mutations, three are nonsense mutations, and one is a putative splicing mutation (Table 1). This identification of *ced-3* null alleles, together with results of genetic analysis of nematodes homozygous for these null mutations in *ced-3*, indicate that, like *ced-4, ced-3* function is not essential to viability. In addition, 10 out of the 12 mutations are clustered in the carboxyl-

terminal region of the gene (exons 6-8, Figure 4B), suggesting that this portion of the encoded protein may be important for activity.

The *ced-3* gene encodes a putative protein of 503 amino acids (Figure 3; SEQ ID NO: 2). The protein is very hydrophilic and no significantly hydrophobic region can be found that might be a transmembrane domain (Figure 5). One region of the Ced-3 protein is very rich in serine (Figure 6A). Comparison of the *C. elegans* protein with the Ced-3 proteins of two related nematodes species, *C. briggsae* and *C. vulgaris*, shows conservation of the serine-rich feature without conservation of the amino acid sequence in this region (Figure 7; SEQ ID NOS: 5 and 6). This suggests that the exact sequence of this serine-rich region may not be important but that the serine-rich feature is. This hypothesis is supported by analysis of *ced-3* mutations: none of 12 EMS-induced *ced-3* mutations is in the serine-rich region (Figure 4B). It is possible that the serine-rich region in Ced-3 is another example of semi-specific protein-protein interaction, similar to acid blobs in transcription factors and basic residues in nuclear localization signals. In all these cases, the exact primary sequence is not important.

The serine-rich region may function as a site for post-translational regulation of Ced-3 activity through protein phosphorylation of the serine residues by a Ser/Thr kinase. McConkey *et al.* (*J. Immunol. 145*:1227-1230 (1990)) have shown that phorbol esters, which stimulate protein kinase C, can block the death of cultured thymocytes induced by exposure to Ca^{++} ionophores or glucocorticoids (Wyllie, *Nature 284*:555-556 (1980); Wyllie *et al., J. Path. 142*:67-77 (1984)). It is possible that protein kinase C may inactivate certain cell death proteins by phosphorylation and, thus, inhibit cell death and promote cell proliferation. Several agents that can elevate cytosolic cAMP levels have been shown to induce thymocyte death, suggesting that protein kinase A may also play a role in

mediating thymocyte death. Further evidence suggests that abnormal phosphorylation may play a role in the pathogenesis of certain cell-degenerative diseases. For example, abnormal phosphorylation of the microtubule-associated protein Tau is found in the brains of Alzheimer's disease and Down's syndrome patients (Grundke-Iqbal *et al., Proc. Natl. Acad. Sci. USA 83*:4913-4917 (1986); Flament *et al., Brain Res. 516*:15-19 (1990)). Thus, it is possible that phosphorylation may have a role in regulating programmed cell death in *C. elegans*. This is consistent with the fairly high levels of *ced-3* and *ced-4* transcripts which suggest that transcriptional regulation alone may be insufficient to regulate programmed cell death.

Structural Relatedness of the *ced-3* and Human Interleukin-1β Convertase Genes and Functional Implications

A search of GenBank, PIR and SWISS-PROT databases using the Blast program (National Center for Biotechnology Information) revealed that human interleukin-1β convertase (ICE) has a 28% amino acid identity with the Ced-3 protein (Figure 6A). A comparable level of overall similarity was found between ICE and the Ced-3 proteins from two other nematode species, *C. briggsae* and *C. vulgaris*.

The carboxyl-terminal regions of Ced-3 and ICE (amino acids 250-503 and amino acids 166-404, respectively) were found to be more conserved (33% identity) than the amino-terminal portions of the two proteins (22% identity). A comparison of human and murine ICEs also indicated a high degree of similarity (80% identity) in the carboxyl-terminal region compared with an overall identity of 62% (Cerretti *et al., Science 256*:97-100 (1992)). Furthermore, deletion analysis of the ICE cDNA sequence has shown that the amino-terminal 119 amino

acids of ICE are not required for enzymatic activity, but that deletions of the carboxyl-terminal region eliminate the enzyme's ability to process pro-IL-1β (Cerretti *et al.*, 1992 *supra*). The observation that most of the inactivating mutations of *ced-3* cluster in the carboxyl-terminal region (Figure 4B) suggests that the activity of Ced-3 also resides (at least partially) in this region. Thus, the identification of the carboxyl-terminal regions of the two proteins as functional domains and the marked similarity of these regions suggest that the Ced-3 and ICE proteins have similar activities, i.e., that ICE has cell death activity similar to Ced-3 and Ced-3 has protease activity similar to ICE.

The possiblity that Ced-3 has protease activity is further supported by the observation that the region surrounding the active cysteine and two autocleavage sites of ICE appear to be conserved in the Ced-3 protein. As shown in Figure 6A, the five amino acids (QACRG, amino acids 283 to 287) surrounding the active cysteine of ICE (Thornberry *et al.*, *Nature 356*:768-774 (1992)) are conserved in amino acids 356 to 360 of Ced-3; this pentapeptide is the longest conserved sequence between ICE and Ced-3. This peptide is also conserved in the Ced-3 proteins of *C. briggsae* and *C. vulgaris* (Figure 7). One inactivating mutation of *ced-3, n2433*, introduces a glycine to serine change near the putative active cysteine (Figure 6A). Example 3 demonstrates that mutations of the active cysteine decrease cell death. Accordingly, one may predict that mutation of the active cysteine in ICE (Cys 285) will yield a therapeutic which decreases cell death.

The human ICE gene encodes a precursor enzyme which is autoproteolytically cleaved at two major sites (amino acids 103 and 297) by the active form of the enzyme (Thornberry *et al.*, 1992 *supra*). The Asp-Ser dipeptides of both autocleavage sites are conserved in Ced-3 (at amino acids 131

and 371) (Figure 6A). The conservation of these functionally important sequences strongly suggests that, like ICE, Ced-3 is a cysteine protease with a similar substrate-specificity. Ced-3 would, therefore, be expected to cleave the IL-1β precursor, as well as other substrates of ICE.

The possibility that ICE is a cell death gene is consistent with evidence which suggests that the production of active IL-1β is involved with cell death processes. Firstly, a variety of studies has suggested that IL-1β can prevent cell death (McConkey *et al.*, *J. Biol. Chem. 265*:3009-3011 (1990); Mangan *et al.*, *J. Immun. 146*:1541-1546 (1991); Sakai *et al.*, *J. Exp. Med. 166*:1597-1602 (1987); Cozzolino *et al.*, *Proc. Natl. Acad. Sci. USA 86*:2369-2373 (1989)). Secondly, active, mature IL-1β appears to be released from cells undergoing cell death. Studies on murine macrophages suggest that release of the active form seems not to be merely due to the lysis of the cells or leaking of cell contents. When murine peritoneal macrophages were stimulated with lipopolysaccharide (LPS) and induced to undergo cell death by exposure to extracellular ATP, mature active IL-1β was released into the culture supernatant. In contrast, when the cells were injured by scraping, IL-1β was released exclusively as the inactive proform (Hogquist *et al.*, *Proc. Natl. Acad. Sci. USA 88*:8485-8489 (1991)).

The similarity between ICE and Ced-3 strongly supports the hypothesis that ICE is involved in cell death. Since Ced-3 is necessary for cell death, one suggestion is that ICE is also necessary for cell death. It is possible that IL-1β can cause cell death. Alternatively, ICE could produce products besides IL-1β, one or more of which can cause cell death. The observation that the ICE transcript is detected in cells that lack IL-1β expression (Cerretti *et al.*, 1992 *supra*) supports this idea. Example 4 demonstrates that known inhibitors of ICE may be

administered to prevent cell death in mammals, particularly motor neuron cell death.

The finding of a human gene related to the nematode *ced-3* gene is consistent with the idea that cell death genes which are structurally related and/or functionally similar to the nematode *ced-3* gene exist in a variety of organisms. This idea is supported by evidence that cell deaths occurring in a variety of organisms, including vertebrates and invertebrates, and possibly microbes and plants, as well as cell deaths observed in various developmental and pathologic situations share a common genetic mechanism. Evidence for this hypothesis is discussed in Example 2. The structural relatedness of ICE suggests that it is a mammalian equivalent of the nematode cell death gene, *ced-3*. The cDNA sequence of ICE is shown in Figure 8 (SEQ ID NO: 3).

The *ced-3*/ICE Gene Family and Uses Thereof

The ICE and *ced-3* genes can be used to isolate additional structurally related genes, including genes from other organisms. Such genes may be identified using probes derived from both the *ced-3* and ICE gene sequences and known techniques, including nucleic acid hybridization, polymerase chain reaction amplification of DNA, screening of cDNA or genomic libraries, and antibody screening of expression libraries. The probes can be all or portions of the genes which are specific to the genes, RNA encoded by the genes, degenerate oligonucleotides derived from the sequences of the encoded proteins, and antibodies directed against the encoded proteins. The sequences of the genes and their protein products can also be used to screen DNA and protein databases for structurally similar genes or proteins.

One strategy for detecting structurally related genes in a number of organisms is to initially probe animals which are taxonomically closely related to the source of the probes, for example, probing other worms with a *ced-3*-derived probe, or probing other mammals with an ICE-derived probe. Closely related species are more likely to possess related genes or gene products which are detected with the probe than more distantly related organisms. Sequences conserved between *ced-3* or ICE and these new genes can then be used to identify similar genes from less closely related species. Furthermore, these new genes provide additional sequences with which to probe the molecules of other animals, some of which may share conserved regions with the new genes or gene products but not with the original probe. This strategy of using structurally related genes in taxonomically closer organisms as stepping stones to genes in more distantly related organisms can be referred to as walking along the taxonomic tree.

Together, *ced-3*, ICE, and related genes obtained as described above would comprise a family of structurally related genes, referred to herein as the *ced-3*/ICE gene family. It is highly likely that at least some of these additional family members would exhibit cell death and/or protease activity. The new genes can be tested for protease activity using known assay methods. For example, the sequence of the protein encoded by a new gene may indicate an active site and substrate-specificity similar to that of ICE, such as observed in Ced-3. This activity can then be verified using the transient expression assays and purified enzyme assays previously described (Cerretti *et al.*, *Science 256*:97-100 (1992); Thornberry *et al.*, *Nature 356*:768-774 (1992)). Cell death activity can be tested in bioassays using transgenic nematodes. A candidate cell death gene, such as the ICE gene, can be injected into Ced-3-deficient mutant animals to determine

whether the gene complements the *ced-3* mutation. Expression libraries can also be screened for cell death genes by this assay.

The *ced-3*, ICE and other related genes which have cell death activity can be used to develop and identify drugs which reduce or increase cell deaths. Drugs which reduce cell deaths are potentially useful for treating diseases and conditions characterized by cell deaths, such as myocardial infarction, stroke, viral and other pathogenic infections (e.g., human immunodeficiency virus), traumatic brain injury, neural and muscular degenerative diseases, and aging. Drugs which cause cell deaths can be used to control or reduce undesired cell populations, such as neoplastic growths and other cancerous cells, infected cells, and cells which produce autoreactive antibodies. Undesired organisms, such as pests, parasites, and recombinant organisms, may also be incapacitated or killed by such drugs.

ICE has been implicated in the growth of certain leukemias (*Sakai et al., J. Exp. Med. 166*:1597 (1987); Cozzolino *et al., Proc. Natl. Acad. Sci. U.S.A. 86*:2369 (1989); Estrov *et al., Blood 78*:1476 (1991); Bradbury *et al., Leukemia 4*:44 (1990); Delwel *et al., Blood 74*:586 (1989); Rambaldi *et al., Blood 78*:3248 (1991)). The observation that the human ICE gene maps to chromosome location 11q23, a site frequently involved in DNA rearrangements seen in human cancers (C. *Cerretti et al., Science 256*: 97-100 (1992)), further suggests that ICE is involved in cancer. The finding that ICE probably functions in cell death implies that ICE and other related genes, like *ced-3*, may be used to develop drugs to control cancerous growth.

In addition, since cell death plays an important role in mammalian hair growth, it seems likely that by controlling cell death, one could cause or prevent hair loss. It has been found that *bcl-2*, a human gene which is structurally related to the gene which prevents cell deaths in nematode development (*ced-9*), is

expressed in the hair follicle in a cell-cycle dependent manner. *ced-9* has been shown to act by antagonizing the activities of the cell death genes, *ced-3* and *ced-4*. Together, these findings suggest that genes equivalent to the *ced-3*, *ced-4*, and *ced-9* genes are involved in the physiology of mammalian hair growth and loss.

Drugs which increase cell deaths may comprise *ced-3*, ICE, and other *ced-3*/ICE family members, their RNA and protein products, constitutively activated mutants of the genes and encoded products, and peptide and non-peptide mimetics of the proteins. Drugs which decrease cell deaths may comprise antisense RNA complementary to the mRNA of a cell death gene, or mutant cell death genes or encoded products, that no longer cause cell death and interfere with the function of wild-type genes. Furthermore, drugs comprising agonists and antagonists of the cell death genes can be designed or identified using the genes or their gene products as targets in bioassays. The bioassays can be conducted in wild-type, mutant, or transgenic nematodes, in which an alteration in programmed cell deaths is an indicator of an effective agonist or antagonist. Bioassays can also be performed in cultured cells transfected with the target cell death gene, into which the substance being tested is introduced. In bioassays for antagonists of cell death, the cultured cells should be put under conditions which induce the activity of the target cell death gene.

Uses of bioassays utilizing *C. elegans* are exemplified by the following:

1) use of normal, wild-type nematodes to screen for drugs or genes that inactivate *ced-3* and hence, prevent programmed cell deaths;

2) use of normal, wild-type nematodes to screen for drugs or genes that activate *ced-3* and hence, cause excess cell deaths;

3) use of mutant nematodes which overexpress *ced-3* or which express a constitutively activated *ced-3* gene to identify drugs or genes that prevent excess cell deaths caused by the *ced-3* mutation;

4) use of mutant nematodes which underexpress *ced-3* or which express an inactivated *ced-3* gene to identify drugs or genes that mimic or complement the *ced-3* mutation;

5) use of transgenic nematodes (with an inactivated endogenous *ced-3* gene) in which either a wild-type or mutant form of ICE or other *ced-3*/ICE family member causes excess cell deaths to identify drugs or genes which antagonize the activity of the transgene; and

6) use of transgenic nematodes which carry a transgene that inhibits cell death (e.g., a transgene that expresses an inhibitory fragment of *ced-3*, ICE or related gene, as described below) to identify drugs that overcome this inhibition and cause cell death.

Drugs can be introduced into nematodes by diffusion, ingestion, microinjection, shooting with a particle gun or other methods. They can be obtained from traditional sources such as extracts (e.g., bacterial, fungal or plant) and compound libraries, or can be provided by newer methods of rationale drug design. Information on functionally important regions of the genes or gene products, gained by sequence comparisons and/or mutational analysis may provide a basis for drug design. Genes can be microinjected into nematodes to produce transgenic nematodes. Individual genes or cDNA and genomic DNA libraries can be screened in this manner.

Agonists and antagonists may also be derived from genes which are not cell death genes, but which interact with, regulate or bypass cell death genes. Such interacting genes may be tested by the bioassays mentioned above, as well as by *in*

vivo genetics in nematodes. In this latter method, interacting genes are identified as secondary mutations which suppress or enhance the *ced-3* mutation. The sequences of these interacting genes can then be used to identify structurally related interacting genes in other organisms.

Similarly, anti-inflammatory drugs may be developed or identified using *ced-3*, ICE and other family members and their encoded products. Drugs which enhance ICE activity may also be useful for boosting the inflammatory response to viral and other infections.

In addition, the availability of a number of structurally related genes makes it possible to carry out structural comparisons. Conserved regions or features of the genes or their encoded products are likely to be functionally significant for cell death and/or protease activity. This information could be helpful in designing or selecting drugs which would mimic or affect the activity of the genes.

Moreover, conservation of functional domains among *ced-3*/ICE family members or their encoded products suggests not only that these genes have similar activities, but that they and their encoded products function via similar mechanisms. This suggests that mutations in conserved regions, mimetics based on conserved regions, and agonists and antagonists which affect the function of conserved regions of one *ced-3*/ICE gene or encoded protein will similarly affect other genes or encoded proteins in the family. This is the rationale behind the use of Ced-3 inhibitors to inhibit ICE and inflammation, and the use of anti-inflammatory drugs which act by inhibiting ICE to inhibit the *ced-3* gene and reduce cell deaths (described further below).

Furthermore, drugs which affect the cell death and/or inflammatory activities of the *ced-3* and ICE genes may also affect other as yet undiscovered

activities of these genes. The biology of IL-1β and ICE is only incompletely understood at the present time, and it is very likely that other functions of both IL-1β and ICE may be discovered. These may include new activities or new physiological processes or diseases in which the respective cytokinetic and proteolytic activities of these molecules are involved. In either case, drugs (such as inhibitory protein portions) which affect ICE activity are likely to affect the new activities and processes, as well.

In addition, mutations and drugs which alter or mimic the activity of one member of the *ced-3*/ICE family can be engineered based on what is known about mutations and drugs affecting another family member with which it shares a conserved region. Mutations in conserved regions which correspond to those found in another family member could be used to produce similar effects. For example, five out of nine inactivating point mutations analyzed in *ced-3* were found to result in alterations of amino acids which are conserved between ICE and Ced-3 (Figure 6A). Amino acid substitutions in ICE corresponding to those in Ced-3 are also expected to result in inactivation (see Example 3). The inhibitory amino-terminal gene portions and constitutively activated carboxyl-terminal gene portions described below are further examples of corresponding mutations which can be made in genes of the *ced-3*/ICE family.

Comparison of Ced-3, ICE, and related proteins also provides insights into the substrate-specificity of ICE and related enzymes. Previous studies on ICE have not identified a consistent consensus cleavage site. A comparison of the Ced-3 and ICE autocleavage sites, together with the cleavage site of pro-IL-1β, reveals that cleavage always occurs after an Asp residue. For this reason, it is likely that Ced-3, ICE, and related proteins are proteases which cleave after some aspartate residues or, perhaps at lower efficiencies, all aspartate residues.

A further use of *ced-3*/ICE family members is to provide diagnostic probes (DNA, RNA, oligonucleotides and antibodies) for diseases involving cell deaths and inflammation in humans and other organisms. It is likely that such diseases are associated with abnormalities in *ced-3*/ICE genes and their gene products. The probes can be used to detect abnormalities in the sequence, level and/or activities of the genes and encoded RNA and protein products. The diseases may be genetic, in which case, the probes may be used in patient and pre-natal testing, or non-genetic, in which case, RNAs and proteins may be examined. In particular, the finding that ICE is a putative cell death gene makes this gene and its derivative molecules potentially useful as diagnostic probes for diseases characterized by cell deaths. Similarly, *ced-3* and its derivative molecules are potentially useful for detecting abnormalities in pathologies in which inflammation is evident. The usefulness of these probes may be multiplied as more genes with known physiological functions are found to be structurally related to *ced-3* and ICE.

Structural Relatedness of *ced-3* and the Murine NEDD-2 Gene

Database searches also revealed that another mammalian protein is similar to the Ced-3 protein (Figure 6B). The murine NEDD-2 (lch-1) protein has 27% amino acid identity and 55% similarity to a carboxyl-terminal portion of Ced-3. The NEDD-2 protein is expressed in the brain of mouse embryos and much less in the murine adult brain; the protein is thought to be involved in the development of the murine central nervous system (Kumar *et al.*, *Biochem. Biophys. Res. Comm. 185*(3):1155-1161 (1992)). The structural similarity between the NEDD-2 and *ced-3* gene products suggests that the NEDD-2 gene is also involved in cell death processes which occur during development, and further supports the

hypothesis that genes which are structurally and functionally related to the nematode *ced-3* gene function in a variety of organisms. Interestingly, the NEDD-2 amino acid sequence is not significantly similar to that of human ICE.

The similarity of the amino acid sequences of Ced-3 and NEDD-2 further suggests that mutations of the NEDD-2 gene which produce alterations in the protein corresponding to alterations in Ced-3 resulting from the mutations, *n1129*, *n1164*, *n2426* and *n1163* (see Figure 6B), will inactivate the NEDD-2 gene.

This invention includes all and portions of the NEDD-2 gene, mutated NEDD-2 genes corresponding to known *ced-3* mutations, RNAs and proteins encoded by the wild-type and mutated genes, and mimetics and other drugs derived from these genes and gene products, which are useful for controlling cell death.

Figures 6C and 6D show alignments of the amino-terminal and carboxyl-terminal regions, respectively, of the Ced-3 proteins of the three nematode species (*C. briggsae*, *C. elegans*, and *C. vulgaris*), the human and murine ICEs and the murine NEDD-2 protein (in 6D only). As shown in these figures (boxed portions), a number of amino acids are completely conserved among these structurally related proteins, and thus, are likely to be important functionally. Mutations of these sites would be expected to alter the activity of the genes.

Inhibitory Portions of the *ced-3* Gene

Fusion constructs containing portions of the *ced-3* gene were found to prevent programmed cell death when expressed in wild-type *C. elegans*. These constructs are represented schematically in Figure 9A. The BGAFQ construct contains a portion of the *ced-3* gene fused 5' of the *E. coli lacZ* gene and another

ced-3 portion fused 3' of *lacZ*. The 5' *ced-3* portion is the genomic sequence from a *Bam*HI site located about 300 base pairs upstream of nucleotide 1 of the sequence shown in Figure 3 to a *Sal*I site at nucleotide 5850. This portion spans sequences 5' of the SL1 acceptor site (nucleotide 2161) to include the 372 codons of the amino-terminal region. The 3' *ced-3* portion of BGAFQ is the genomic sequence from a *Not*I site at nucleotide 5927 in the *ced-3* gene to an *Apa*I site located about 1.5 kb downstream of nucleotide 7653 of the sequence in Figure 3. This portion contains the carboxyl-terminal codons from 398 to the end (codon 503) and 3' untranslated sequences.

The PBA construct has a smaller portion of the *ced-3* gene which is the genomic sequence from the same *Bam*HI site as in BGAFQ to a *Bgl*II site at nucleotide 3020 (Figure 9A) fused 5' of the *lacZ* gene. This *ced-3* portion spans sequences 5' of the SL1 acceptor site to include the first 149 codons of the amino-terminal region.

Both constructs were made using the pBluescript vector (Stratagene) and fragments containing the *lacZ* construct from the pPD vectors of Fire (*EMBO J. 5*:2673-2690 (1986)). The *lacZ*-containing portion has the entire *lacZ* coding sequence except for the first 11 codons. In addition, there is a synthetic intron and a nuclear localization signal upstream of the *lacZ* gene and a fragment of the 3' end of the *unc-54* gene downstream of the *lacZ* gene (Figure 9B). Construct PBA was made by inserting a *Bam*HI-*Apa*I fragment containing the *lacZ* construct shown in Figure 9B from Andy Fire's vector, pPD22.04, into the *Bgl*II-*Apa*I fragment of the *ced-3*-containing plasmid, pJ40. Construct BGAFQ was made by inserting a *Sal*I-*Eag*I fragment containing the same *lacZ* construct from pPD22.04 into the *Sal*I-*Not*I fragment of pJ40A, which is pJ40 without the *Not*I site in the vector.

Table 2 shows the results of injecting wild-type nematodes with the two constructs. These results indicate that the BGAFQ and PBA fusion constructs prevent cell deaths which normally occur in the development of the nematodes. These fusion constructs were further observed to prevent cell deaths and the apparently associated inviability caused by a loss-of-function mutation in *ced-9*, a gene which functions to keep certain cells from dying during nematode development, and which has been shown to act by antagonizing *ced-3* and second cell death gene, *ced-4*.

Both constructs express β-galactosidase activity in wild-type nematodes. Since the pBluescript vector does not contain eukaryotic transcriptional or translational start sites, these signals are probably supplied by the *ced-3* gene portions fused 5' of *lacZ*. Furthermore, since the PBA construct works to prevent cell death, it seems that the *ced-3* portion in BGAFQ needed for inhibition is the portion fused upstream of *lacZ* (as opposed to the portion located downstream of *lacZ*). Presumably, only the region from the *Bam*HI site to nucleotide 3020 is needed in BFAGQ, since this is all that is contained in PBA.

A construct that contains the PBA *ced-3* portion but not any of the *lacZ* portion did not prevent cell death, suggesting that fusion to portions of *lacZ* is needed for expression or action of the inhibitory gene portion.

These observations indicate that the amino-terminal portion of the Ced-3 protein, possibly in conjunction with a portion of *E. coli* β-galactosidase, can act to prevent programmed cell deaths in *C. elegans*. One plausible mechanism is that this portion of the Ced-3 protein acts in a dominant negative or antimorphic fashion, to prevent the activity of the normal Ced-3 protein. (It is known that inactivation of the Ced-3 protein results in an absence of programmed cell deaths.) Such dominant negative activity could be a result of the partial Ced-3 protein

binding to and, thereby, inactivating the normal Ced-3 protein; consistent with this model is the finding that the active form of the structurally similar ICE protein is dimeric. Alternatively, the partial Ced-3 protein may bind to a molecule with which the normal Ced-3 protein must interact to function and by preventing this interaction, inhibits Ced-3 activity.

Due to the structural similarity of ICE to the Ced-3 protein, fusion constructs encoding amino-terminal portions of ICE would also be expected to inhibit the activity of the *ced-3* gene. In particular, those portions of the ICE gene corresponding to the *ced-3* gene portions in BGAFQ and PBA, i.e., ICE codons 1 to 298 and codons 1 to 111, or active subportions of these, are expected to inhibit *ced-3*. A further extension of this reasoning suggests that corresponding gene portions of any structurally related *ced-3*/ICE family member would also have an inhibitory effect on *ced-3* activity.

Furthermore, the structural relatedness of the *ced-3* and ICE genes implies that the ICE enzyme could also be inhibited by fusion constructs containing amino-terminal portions of the ICE gene, as well as corresponding portions of other structurally related genes, such as *ced-3*.

Identification of portions of the *ced-3*, ICE, and related genes which inhibit the *ced-3* gene can be carried out by testing expression constructs containing these gene portions or their encoded products in bioassays for cell death activity. Identification of gene portions or encoded products which inhibit ICE can be carried out using previously described assays for ICE activity. For example: 1) wild-type worms can be injected with portions of the *ced-3* or other structurally related gene, such as ICE, to determine if they prevent programmed cell death; 2) portions of the ICE protein or other structurally similar protein, such as Ced-3, can be co-expressed with ICE and pro-IL-1β in nematodes or cultured mammalian

cells to see if they inhibit ICE-catalyzed cleavage of the IL-1β precursor; and 3) peptides or nucleic acids containing portions of the amino acid or coding sequence of ICE or similar protein, such as Ced-3, can be tested using purified ICE and synthetic substrates.

Inhibitory portions of the *ced-3* gene, ICE, and structurally related genes, their encoded RNAs and proteins, and peptide and non-peptide mimetics of the proteins may be used to reduce cell deaths and/or inflammation, and are, thus, useful for the treatment of diseases involving these processes. The encoded proteins and peptide and non-peptide mimetics can be delivered by various known methods and routes of drug delivery. For example, they can be administered orally or by another parenteral route or by a non-parenteral route (e.g., by injection intramuscularly, intraperitoneally or intravenously or by topical administration). Alternatively, expression constructs containing the gene portions can be made using heterologous transcriptional and translational signals or signals native to the gene portions. The constructs can be delivered into cells by various methods of gene therapy, such as retroviral infection. These constructs (and any other constructs which encode activity decrease cell death) may be used for example, to prevent localized cell death at the site of organ and tissue transplantation.

Interestingly, those ICE gene portions corresponding to the *ced-3* portions of BGAFQ and PBA encode approximately the protein fragments which result from cleavage at each of the two autocleavage sites (amino acids 103 and 297). This observation suggests that autoproteolytic conversion of the proenzyme to active ICE involves cleaving off the inhibitory amino-terminal portions of the protein. Active ICE is a heterodimer composed of subunits of about 20 and 10 kilodaltons (Thornberry *et al.*, *Nature 356*:768-774 (1992)). These subunits have been shown to be derived from the ICE proenzyme and correspond to amino acids

120 to 297 (p20) and 317 to 404 (p10). Kinetic studies suggest that association of the two subunits is required for activity of the enzyme. It is possible that the amino-terminal region of the protein interferes with this association.

This implies that mutant proteins in which the inhibitory amino-terminal regions are deleted may be constitutively activated. Thus, carboxyl-terminal portions of the Ced-3, ICE, and related proteins, and constructs and RNAs expressing these portions, are potentially useful for increasing cell deaths and/or IL-1β production. Constructs which may be used include those which express the carboxyl region of ICE, which encodes the two subunits of the active enzyme, as well as those which express each of these subunits separately. In addition, it is possible that the amino region of ICE, which is not needed for ICE enzymatic activity *in vitro*, is important for ICE activity or the regulation of ICE activity *in vivo*. Consistent with this idea is the finding that two of the *ced-3* mutations map in this region. For this reason, a construct which expresses the amino region of Ced-3, ICE or a Ced-3/ICE gene family member may also be used. Furthermore, the NEDD-2 protein, which is similar to a carboxyl-terminal portion of the Ced-3 portion, may also exhibit constitutive activity in causing cell deaths. Thus, all or active portions of NEDD-2, and DNA and RNA encoding NEDD-2 proteins, would be expected to produce cell death activity when expressed. Drugs comprising activated molecules derived from the carboxyl-terminal regions of Ced-3, ICE and other proteins of the Ced-3/ICE family and from the NEDD-2 protein, DNAs and RNAs encoding these proteins and protein fragments, as well as peptide and non-peptide mimetics, are potentially useful for controlling or reducing the size of undesirable cell populations, such as cancerous cells, infected cells, cells producing autoreactive antibodies and hair follicle cells. Such drugs may also be useful for incapacitating or killing undesired organisms, such as

parasites, pests, and genetically engineered organisms. For example, a number of nematodes are human, animal and plant parasites.

ICE Inhibitors As Inhibitors of Cell Death

The conservation of the active site of ICE (active cysteine and surrounding amino acids) in the Ced-3 protein implies that Ced-3 is a cysteine protease which interacts with its substrate by a similar mechanism. Hence, it is likely that inhibitors of ICE which interfere with this mechanism, or chemical analogs of these inhibitors, will inhibit Ced-3 function and inhibit cell death resulting from ICE activity.

Peptide aldehydes containing the ICE recognition site:

P4--P3--P2--P1
Tyr-Val-Ala-Asp (SEQ ID NO: 17)

or a substituted site in which P2 is Ala, His, Gln, Lys, Phe, Cha, or Asp, have been shown to be effective, specific, and reversible inhibitors of the protease activity of ICE (Thornberry *et al.*, *Nature 356*:768-774 (1992)). These molecules are thought to act as transition analogs, which compete for ICE binding to its substrate, pro-IL-1β. Three such inhibitors have been described: Inhibitor B (Ac-Tyr-Val-Ala-Asp-CHO; SEQ ID NO: 16); Inhibitor C (Ac-Tyr-D-Ala-Ala-Asp-CHO; SEQ ID NO: 19); and Inhibitor D (Ac-Tyr-Val-Lys-Asp-CHO). Of these, Inhibitor B is the most potent, with a K_i=0.76 nM compared to K_i=3 nM for D and K_i=1.5 μM for C. Example 4 provides evidence that these inhibitors may be used to prevent cell deaths in mammals.

In addition, the *crmA* gene of cowpox virus has been found to encode a serpin which specifically inhibits ICE (Ray *et al.*, *Cell 69*:597-604 (1992)). The serpin acts by preventing the proteolytic activation of ICE. This inhibitor of ICE is

also expected to inhibit structurally similar proteins, such as Ced-3. The *crmA* gene and methods for obtaining purified CrmA protein have been described (Pickup *et al., Proc. Natl. Acad. Sci. USA 83*:7698-7702 (1986); Ray *et al.*, 1992 *supra*). This invention includes the use of inhibitors of ICE, such as peptide aldehydes, and particularly inhibitor B, and the CrmA protein, as drugs for decreasing the activity of cell death genes and, thus, for treatment of diseases characterized by cell deaths.

The following examples illustrate the invention and are not intended to be limiting in any way.

EXAMPLE 1

CLONING, SEQUENCING, AND CHARACTERIZATION OF THE *CED-3* GENE

MATERIALS AND METHODS

General Methods and Strains

The techniques used for the culturing of *C. elegans* were as described by Brenner (*Genetics* 77:71-94 (1974)). All strains were grown at 20°C. The wild-type parent strains were *C. elegans* variety Bristol strain N2, Bergerac strain EM1002 (Emmons *et al.*, *Cell 32*:55-65 (1983)), *C. briggsae* and *C. vulgaris* (obtained from V. Ambros). The genetic markers used are described below. These markers have been described by Brenner (1974 *supra*), and Hodgkin *et al.* (In: *The Nematode Caenorhabditis elegans*, Wood and the Community of *C. elegans* Researchers (eds.), Cold Spring Harbor Laboratory, 1988, pp 491-584). Genetic nomenclature follows the standard system (Horvitz *et al.*, *Mol. Gen. Genet. 175*:129-133 (1979)):

LG I: *ced-1(e1375); unc-54(r323)*

LG VI: *unc-31(e928), unc-30(e191), ced-3(n717, n718, n1040, n1129, n1163, n1164, n1165, n1286, n1949, n2426, n2430, n2433), unc-26(e205), dpy-4(e1166)*

LG V: *egl-1(n986); unc-76(e911)*

LG X: *dpy-3(e27)*

Isolation of Additional Alleles of *ced-3*

A non-complementation screen was designed to isolate new alleles of *ced-3*. Because animals heterozygous for *ced-3(n717)* in *trans* to a deficiency are viable (Ellis and Horvitz, *Cell 44*:817-829 (1986)), animals carrying a complete loss-of-function *ced-3* allele generated by mutagenesis were expected to be viable *in trans* to *ced-3(n717)*, even if the new allele was inviable in homozygotes. Fourteen EMS mutagenized *egl-1* males were mated with *ced-3(n717) unc-26(e205); egl-1(n487); dpy-3(e27)* hermaphrodites. *egl-1* was used as a marker in this screen. Dominant mutations in *egl-1* cause the two hermaphrodite specific neurons, the HSNs, to undergo programmed cell death (Trent *et al.*, *Genetics 104*:619-647 (1983)). The HSNs are required for normal egg-laying, and egl-1(n986) hermaphrodites, which lack HSNs, are egg-laying defective (Trent *et al.*, 1983 *supra*). The mutant phenotype of *egl-1* is suppressed in a *ced-3; egl-1* strain because mutations in *ced-3* block programmed cell deaths. *egl-1* males were mutagenized with EMS and crossed with *ced-3(n717), unc-26(e205); egl-1(n487); dpy-3(e27)*. Most cross progeny were egg-laying defective because they were heterozygous for *ced-3* and homozygous for *egl-1*. Rare egg-laying competent animals were picked as candidates for carrying new alleles of *ced-3*. Four such animals were isolated from about 10,000 F1 cross progeny of EMS-mutagenized animals. These new mutations were made homozygous to confirm that they carried recessive mutations of *ced-3*.

Molecular Biology

Standard techniques of molecular biology were used (Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1983).

Two cosmid libraries were used extensively in this work: a *Sau*3AI partial digest genomic library of 7000 clones in the vector pHC79 and a *Sau*3AI partial digest genomic library of 6000 clones in the vector pJB8 (Ish-Horowicz and Burke, *Nucleic Acids Res. 9*:2989 (1981)). The "right" end of MMM-C1 was cloned by cutting it with *Hin*dIII and self-ligating. The "left" end of MMM-C1 was cloned by cutting it with *Bgl*II or *Sal*I and self-ligating.

The "right" end of Jc8 was made by digesting Jc8 with *Eco*RI and self-ligating. The "left" end of Jc8 was made by digesting Jc8 by *Sal*I and self-ligating.

C. elegans RNA was extracted using guanidine isothiocyanate (Kim and Horvitz, *Genes & Dev. 4*:357-371 (1990)). Poly(A)$^+$ RNA was selected from total RNA by a poly(dT) column (Maniatis et al., 1983 *supra*). To prepare stage-synchronized animals, worms were synchronized at different developmental stages (Meyer and Casson, *Genetics 106:29-44* (1986)).

For DNA sequencing, serial deletions were made according to a procedure developed by Henikoff (*Gene 28*:351-359 (1984)). DNA sequences were determined using Sequenase and protocols obtained from US Biochemicals with minor modifications.

The Tc1 DNA probe for Southern blots was pCe2001, which contains a Bergerac Tc1 element (Emmons *et al., Cell 32*:55-65 (1983)). Enzymes were purchased from New England Biolabs, and radioactive nucleotides were from Amersham.

Primer extension procedures followed the protocol by Robert E. Kingston (In: *Current Protocols in Molecular Biology*, Ausubel *et al.* (eds.), Greene Publishing Associates and Wiley-Interscience, New York, p. 4.8.1) with minor modifications.

Polymerase chain reaction (PCR) was carried out using standard protocols supplied by the GeneAmp Kit (Perkin Elmer). The primers used for primer extension and PCR are as follows:

Pex2: 5' TCATCGACTTTTAGATGACTAGAGAACATC 3' (SEQ ID NO: 7);

Pex1: 5' GTTGCACTGCTTTCACGATCTCCCGTCTCT 3' (SEQ ID NO: 8);

SL1: 5' GTTTAATTACCCAAGTTTGAG 3' (SEQ ID NO: 9);

SL2: 5' GGTTTTAACCAGTTACTCAAG 3' (SEQ ID NO: 10);

Log5: 5' CCGGTGACATTGGACACTC 3' (SEQ ID NO: 11); and

Oligo10: 5' ACTATTCAACACTTG 3' (SEQ ID NO: 12).

Germline Transformation

The procedure for microinjection basically follows that of A. Fire (*EMBO J. 5*:2673-2680 (1986)) with modifications: Cosmid DNA was twice purified by CsCl-gradient. Miniprep DNA was used when deleted cosmids were injected. To prepare miniprep DNA, DNA from 1.5 ml overnight bacterial culture in superbroth (12 g Bacto-tryptone, 24 g yeast extract, 8 ml 50% glycerol, 900 ml H_2O, autoclaved; after autoclaving, 100 ml 0.17 M KH_2PO_4 and 0.72 M KH_2PO_4 were added) was extracted by alkaline lysis method as described in Maniatis et al. (1983 *supra*). DNA was treated with RNase A (37°, 30 minutes) and then with protease K (55°, 30 minutes), extracted with phenol and then chloroform, precipitated twice (first in 0.3 M sodium acetate and second in 0.1 M potassium acetate, pH 7.2), and resuspended in 5 µl injection buffer as described by A. Fire

(1986 *supra*). The DNA concentration for injection is in the range of 100 ug to 1 mg per ml.

All transformation experiments used *ced-1(e1735)*; *unc-31(e928) ced-3(n717)* strain. *unc-31* was used as a marker for co-transformation (Kim and Horvitz, 1990 *supra*). *ced-1* was present to facilitate scoring of the Ced-3 phenotype. The mutations in *ced-1* block the engulfment process of cell death, which makes the corpses of the dead cells persist much longer than in wild-type animals (Hedgecock *et al.*, *Science 220*:1277-1280 (1983)). The Ced-3 phenotype was scored as the number of dead cells present in the head of young L1 animals. The cosmid C10D8 or the plasmid subclones of C10D8 were mixed with C14G10 (unc-31(+)-containing) at a ratio of 2:1 or 3:1 to increase the chances that a Unc-31(+) transformant would contain the cosmid or plasmid being tested as well. Usually, 20-30 animals were injected in one experiment. Non-Unc F1 progeny of the injected animal were isolated three to four days later. About 1/2 to 1/3 of the non-Unc progeny transmitted the non-Unc phenotype to F2 progeny and established a transformant line. The young L1 progeny of such non-Unc transformant were checked for the number of dead cells present in the head using Nomarski optics.

RESULTS

Isolation of Additional *ced-3* Alleles

All of the *ced-3* alleles that existed previously were isolated in screens designed to detect viable mutants displaying the Ced phenotype (Ellis and Horvitz, *Cell 44*:817-829 (1986)). Such screens may have systematically missed any class of *ced-3* mutations that is inviable as homozygotes. For this reason, a scheme was

designed that could isolate recessive lethal alleles of *ced-3*. Four new alleles of *ced-3 (n1163, n1164, n1165, n1286)* were isolated in this way. Since new alleles were isolated at a frequency of about 1 in 2500, close to the frequency expected for the generation of null mutations by EMS in an average *C. elegans* gene (Brenner, *Genetics 77*:71-94 (1974); Greenwald and Horvitz, *Genetics 96*:147-160 (1980)), and all four alleles are homozygous viable, it was concluded that the null allele of *ced-3* is viable.

Mapping RFLPs near *ced-3*

Tc1 is a *C. elegans* transposable element that is thought to be immobile in the common laboratory Bristol strain and in the Bergerac strain (Emmons *et al.*, *Cell 32*:55-65 (1983)). In the Bristol strain, there are 30 copies of Tc1, while in the Bergerac strain, there are more than 400 copies of Tc1 (Emmons et al., 1983 *supra*; Finney, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, Massachusetts, 1987). Because the size of the *C. elegans* genome is small (haploid genome size 8 x 10^7 bp) (Sulston and Brenner, *Genetics 77*:95-104 (1976)), a polymorphism due to Tc1 between the Bristol and Bergerac strains would be expected to occur about once every 200 kb. Restriction fragment length polymorphisms (RFLPs) can be used as genetic markers and mapped in a manner identical to conventional mutant phenotypes. A general scheme has been designed to map Tc1 elements that are dimorphic between the Bristol and Bergerac strains near any gene of interest (Ruvkun *et al.*, *Genetics 121*:501-516 (1989)). Once tight linkage of a particular Tc1 to a gene of interest has been established, that Tc1 can be cloned and used to initiate chromosome walking.

A 5.1 kb Bristol-specific Tc1 *Eco*RI fragment was tentatively identified as containing the Tc1 closest to *ced-3*. This Tc1 fragment was cloned using

cosmids from a set of Tc1-containing *C. elegans* Bristol genomic DNA fragments. DNA was prepared from 46 such Tc1-containing cosmids and screened using Southern blots to identify the cosmids that contain a 5.1 kb *Eco*RI Tc1-containing fragment. Two such cosmids were identified: MMM-C1 and MMM-C9. The 5.1 kb *Eco*RI fragment was subcloned from MMM-C1 into pUC13 (Promega). Since both ends of Tc1 contain an *Eco*RV site (Rosenzweig *et al.*, *Nucleic Acids Res. 11*:4201-4209 (1983)), *Eco*RV was used to remove Tc1 from the 5.1 kb *Eco*RI fragment, generating a plasmid that contains only the unique flanking region of this Tc1-containing fragment. This plasmid was then used to map the specific Tc1 without the interference of other Tc1 elements.

unc-30(e191) ced-3(n717) dpy-4(e1166)/+++ males were crossed with Bergerac (EM1002) hermaphrodites, and Unc non-Dpy or Dpy non-Unc recombinants were picked from among the F2 progeny. The recombinants were allowed to self-fertilize, and strains that were homozygous for either *unc-30(e191) dpy-4(Bergerac)* or *unc-30(Bergerac) dpy-4(e1166)* were isolated. After identifying the *ced* genotypes of these recombinant strains, DNA was prepared from these strains. A Southern blot of DNA from these recombinants was probed with the flanking sequence of the 5.1 kb *Eco*RI Tc1 fragment. This probe detects a 5.1 kb fragment in Bristol N2 and a 3.4 kb fragment in Bergerac. Five out of five *unc-30 ced-3 dpy(+Berg)* recombinants, and one of one *unc-30(+Berg) ced-3 dpy-4* recombinants showed the Bristol pattern. Nine of ten *unc-30(+Berg) dpy-4* recombinants showed the Bergerac pattern. Only one recombinant of *unc-30(+Berg) dpy-4* resulted from a cross-over between *ced-3* and the 5.1 kb Tc1 element. The genetic distance between *ced-3* and *dpy-4* is 2 map units (mu). Thus, this Tc1 element is located 0.1 mu on the right side of *ced*-3.

Cosmids MMM-C1 and MMM-C9 were used to test whether any previously mapped genomic DNA cosmids overlapped with these two cosmids. A contig of overlapping cosmids was identified that extended the cloned region near *ced-3* in one direction.

To orient MMM-C1 with respect to this contig, both ends of MMM-C1 were subcloned and these subclones were used to probe the nearest neighboring cosmid C48D1. The "right" end of MMM-C1 does not hybridize to C48D1, while the "left" end does. Therefore, the "right" end of MMM-C1 extends further away from the contig. To extend this contig, the "right" end of MMM-C1 was used to probe the filters of two cosmid libraries (Coulson *et al., Proc. Natl. Acad. Sci. USA 83*:7821-7825 (1986)). One clone, Jc8, was found to extend MMM-C1 in the opposite direction of the contig.

RFLPs between the Bergerac and Bristol strains were used to orient the contig with respect to the genetic map. Bristol (N2) and Bergerac (EM1002) DNA was digested with various restriction enzymes and probed with different cosmids to look for RFLPs. Once such an RFLP was found, DNA from recombinants of the Bristol and Bergerac strains between *ced-3* and *unc-26*, and between *unc-30* and *ced-3* was used to determine the position of the RFLP with respect to *ced-3*.

The "right" end of Jc8, which represents one end of the contig, detects an RFLP *(nP33)* when N2 and EM1002 DNA was digested with *Hin*dIII. A Southern blot of DNA from recombinants between three *ced-3(+Berg) unc-26* was probed with the "right" end of Jc8. Three of three *+Berg unc-26* recombinants showed the Bristol pattern, while two of two *ced-3 unc-26(+Berg)* recombinants showed the Bergerac pattern. Thus, *nP33* mapped very close or to the right side of *unc-26*.

The "left" end of Jc8 also detects a *Hin*dIII RFLP *(nP34)*. The same Southern blot was reprobed with the Jc8 "left" end. Two of the two *ced-3 unc-26(+Berg)* recombinants and two of the three *ced-3(+Berg) unc-26* recombinants showed the Bergerac pattern. One of the three *ced-3(+Berg) unc-26* recombinants showed the Bristol pattern. The genetic distance between *ced-3* and *unc-26* is 0.2 mu. Thus, *nP34* was mapped between *ced-3* and *unc-26*, about 0.1 mu on the right side of *ced-3*.

The flanking sequence of the 5.1 kb *Eco*RI Tc1 fragment (named *nP35*) was used to probe the same set of recombinants. Two of three *ced-3(+Berg) unc-26* recombinants and two of two *ced-3 unc-26(+Berg)* recombinants showed the Bristol pattern. Thus, *nP35* was also found to be located between *ced-3* and *unc-26*, about 0.1 mu on the right side of *ced-3*.

A similar analysis using cosmid T10H5 which contains the *Hin*dIII RFLP *(nP36)*, and cosmid B0564, which contains a *Hin*dIII RFLP (*nP37*), showed that *nP36* and *nP37* mapped very close or to the right of *unc-30*.

These experiments localized the *ced-3* gene to an interval of three cosmids. The positions of the RFLPs, and of *ced-3, unc-30* and *unc-26* on chromosome IV, and their relationships to the cosmids are shown in Figure 1. It has been demonstrated by microinjection that cosmids C37G8 and C33F2 carry the *unc-30* gene (John Sulston, personal communication). Thus, the region containing the *ced-3* gene was limited to an interval of two cosmids. These results are summarized in Figure 1.

Complementation of *ced-3* by Germline Transformation

Cosmids that were candidates for containing the ced-3 gene were microinjected into a *ced-3* mutant to see if they rescue the mutant phenotype. The

procedure for microinjection was that of A. Fire (*EMBO J. 5*:2673-2680 (1986)) with modifications. *unc-31*, a mutant defective in locomotion, was used as a marker for cotransformation (Kim and Horvitz, *Genes & Dev. 4*:357-371 (1990)), because the phenotype of *ced-3* can be examined only by using Nomarski optics. Cosmid C14G10 (containing *unc-31*(+)) and a candidate cosmid were coinjected into *ced-1(e1375)*; *unc-31(e928) ced-3(n717)* hermaphrodites, and F1 non-Unc transformants were isolated to see if the non-Unc phenotype could be transmitted and established as a line of transformants. Young L1 progeny of such transformants were examined for the presence of cell deaths using Nomarski optics to see whether the Ced-3 phenotype was suppressed. Cosmid C14G10 containing *unc-31* alone does not rescue *ced-3* activity when injected into a *ced-3* mutant. Table 4 summarizes the results of these transformation experiments.

As shown in Table 3, of the three cosmids injected (C43C9, W07H6 and C48D1), only C48D1 rescued the Ced-3 phenotype (2/2 non-Unc transformants rescued the Ced-3 phenotype). One of the transformants, *nEX2*, appears to be rescued by an extra-chromosomal array of injected cosmids (Way and Chalfie, *Cell 54*:5-16 (1988)), which is maintained as an unstable duplication, since only 50% of the progeny of a non-Unc Ced(+) animal are non-Unc Ced(+). Since the non-Unc Ced(+) phenotype of the other transformant (nIS1) is transmitted to all of its progeny, it is presumably an integrated transformant. L1 *ced-1* animals contain an average of 23 cell corpses in the head. L1 *ced-1*; *ced-3* animals contain an average of 0.3 cell corpses in the head. *ced-1*; *unc-31 ced-3; nIS1* and *ced-1; unc-31 ced-3; nEX2* animals contain an average of 16.4 and 14.5 cell corpses in the head, respectively. From these results, it was concluded that C48D1 contains the *ced-3* gene.

In order to locate *ced-3* more precisely within the cosmid C48D1, this cosmid was subcloned and the subclones were tested for the ability to rescue *ced-3* mutants. C48D1 DNA was digested with restriction enzymes that cut rarely within the cosmid and the remaining cosmid was self-ligated to generate a subclone. Such subclones were then injected into a *ced-3* mutant to look for completion. When C48D1 was digested with *Bam*HI and self-ligated, the remaining 14 kb subclone (named C48D1-28) was found to rescue the Ced-3 phenotype when injected into a *ced-3* mutant (Figure 2 and Table 4). C48D1-28 was then partially digested with *Bgl*II and self-ligated. Clones of various lengths were isolated and tested for their ability to rescue *ced-3*.

One clone, C48D1-43, which did not contain a 1.7 kb *Bgl*II fragment of C48D1-28, was able to rescue *ced-3* (Figure 2 and Table 4). C48D1-43 was further subcloned by digesting with *BamHI* and *ApaI* to isolate a 10 kb *Bam*HI-*Apa*I fragment. This fragment was subcloned into pBSKII+ to generate pJ40. pJ40 can restore Ced-3+ phenotype when microinjected into a *ced-3* mutant. pJ40 was subcloned by deleting a 2 kb *Bgl*II-*Apa*I fragment to generate pJ107. pJ107 was also able to rescue the Ced-3 phenotype when microinjected into a *ced-3* mutant. Deletion of 0.5 kb on the left side of pJ107 could be made by *Exo*III digestion (as in pJ107del28 and pJ107del34) without affecting Ced-3 activity; in fact, one transgenic line, *nEX17*, restores full Ced-3 activity. However, the *ced-3* rescuing ability was significantly reduced when 1 kb was deleted on the left side of pJ107 (as in pJ107del12 and pJ107del27), and the ability was completely eliminated when a 1.8 kb *Sal*I-*Bgl*II fragment was deleted on the right side of pJ107 (as in pJ55 and pJ56), suggesting that this *Sal*I site is likely to be in the *ced-3* coding region. From these experiments, *ced-3* was localized to a DNA fragment of 7.5 kb. These results are summarized in Figure 2 and Table 4.

ced-3 Transcript

pJ107 was used to probe a Northern blot of N2 RNA and detected a band of 2.8 kb. Although this transcript is present in 12 *ced-3* mutant animals, subsequent analysis showed that all 12 *ced-3* mutant alleles contain mutations in the genomic DNA that codes for this mRNA (see below), thus establishing this RNA as a *ced-3* transcript.

The developmental expression pattern of *ced-3* was determined by hybridizing a Northern blot of RNA from animals of different stages (eggs, L1 through L4 larvae and young adult) with the *ced-3* cDNA subclone pJ118. Such analysis revealed that the *ced-3* transcript is most abundant during embryonic development, which is the period when most programmed cell deaths occur, but it was also detected during the L1 through L4 larval stages and is present in relatively high levels in young adults. This result suggests that *ced-3* is not only expressed in cells undergoing programmed cell death.

Since *ced-3* and *ced-4* are both required for programmed cell death in *C. elegans*, one of the genes might act as a regulator of transcription of the other gene. To examine if *ced-4* regulates the transcription of *ced-3*, RNA was prepared from eggs of *ced-4* mutants (*n1162, n1416, n1894*, and *n1920*), and a Northern blot was probed with the *ced-3* cDNA subclone pJ118. The presence of RNA in each lane was confirmed with an actin I probe. Such an experiment showed that the level of *ced-3* transcript is normal in *ced-4* mutants. This indicates that *ced-4* is unlikely to be a transcriptional regulator of *ced-3*.

Isolation of a *ced-3* cDNA

To isolate cDNA of *ced-3*, pJ40 was used as a probe to screen a cDNA library of N2 (Kim and Horvitz, *Genes & Dev. 4*:357-371 (1990)). Seven cDNA

clones were isolated. These cDNAs can be divided into two groups: one is 3.5 kb and the other 2.5 kb. One cDNA from each group was subcloned and analyzed further. pJ85 contains the 3.5 kb cDNA. Experiments showed that pJ85 contains a *ced-3* cDNA fused to an unrelated cDNA; on Northern blots of N2 RNA, the pJ85 insert hybridizes to two RNA transcripts, and on Southern blots of N2 DNA, pJ85 hybridizes to one more band than pJ40 (*ced-3* genomic DNA) does. pJ87 contains the 2.5 kb cDNA. On Northern blots, pJ87 hybridizes to a 2.8 kb RNA and on Southern blots, it hybridizes only to bands to which pJ40 hybridizes. Thus, pJ87 contains only *ced-3* cDNA.

To show that pJ87 does contain the *ced-3* cDNA, a frameshift mutation was made in the *Sal*I site of pJ40 corresponding to the *Sal*I site in the pJ87 cDNA. Constructs containing the frameshift mutation failed to rescue the Ced-3 phenotype when microinjected into *ced-3* mutant animals, suggesting that *ced-3* activity has been eliminated.

<u>*ced-3* Sequence</u>

The DNA sequence of pJ87 was determined (Figure 3). pJ87 contains an insert of 2.5 kb which has an open reading frame of 503 amino acids (Figure 3; SEQ ID NO: 2). The 5' end of the cDNA contains 25 bp of poly-A/T sequence, which is probably an artifact of cloning and is not present in the genomic sequence. The cDNA ends with a poly-A sequence, suggesting that it contains the complete 3' end of the transcript. 1 kb of pJ87 insert is untranslated 3' region and not all of it is essential for ced-3 expression, since genomic constructs with deletions of 380 bp of the 3' end can still rescue *ced-3* mutants (pJ107 and its derivatives, see Figure 2).

To confirm the DNA sequence obtained from the *ced-3* cDNA and to study the structure of the *ced-3* gene, the genomic sequence of the *ced-3* gene in the plasmid pJ107 was determined (Figure 3; SEQ ID NO: 1). Comparison of the *ced-3* genomic and cDNA sequences revealed that the *ced-3* gene has seven introns that range in size from 54 bp to 1195 bp (Figure 4A). The four largest introns, as well as sequences 5' of the start codon, were found to contain repetitive elements (Figure 3). Five types of repetitive elements were found, some of which have been previously characterized in non-coding regions of other *C. elegans* genes, such as *fem-1* (Spence *et al.*, *Cell 60*:981-990 (1990)), *lin-12* (J. Yochem, personal communication), and *myoD* (Krause *et al.*, *Cell 63*:907-919 (1990)). Of these, repeat 1 was also found in *fem-1* and *myoD*, repeat 3 in *lin-12* and *fem-1*, repeat 4 in *lin-12*, and repeats 2 and 5 were novel repetitive elements.

A combination of primer extension and PCR amplification was used to determine the location and nature of the 5' end of the *ced-3* transcript. Two primers (Pex1 and Pex2) were used for the primer extension reaction. The Pex1 reaction yielded two major bands, whereas the Pex2 reaction gave one band. The Pex2 band corresponded in size to the smaller band from the Pex1 reaction, and agreed in length with a possible transcript that is trans-spliced to a *C. elegans* splice leader (Bektesh, *Genes & Devel. 2*:1277-1283 (1988)) at a consensus splice acceptor at position 2166 of the genomic sequence (Figure 3). The nature of the larger Pex1 band is unclear.

To confirm the existence of this trans-spliced message in wild-type worms, total *C. elegans* RNA was PCR amplified using the SL1-Log5 and SL2-Log5 primer pairs, followed by a reamplification using the SL1-Oligo10 and SL2-Oligo10 primer pairs. The SL1 reaction yielded a fragment of the predicted length. The identity of this fragment was confirmed by sequencing. Thus, at least

some, if not most, of the *ced-3* transcript is trans-spliced to SL1. Based on this result, the start codon of the *ced-3* message was assigned to the methionine encoded at position 2232 of the genomic sequence (Figure 3).

The DNA sequences of 12 EMS-induced *ced-3* alleles were also determined (Figure 3 and Table 1). Nine of the 12 are missense mutations. Two of the 12 are nonsense mutations, which might prematurely terminate the translation of *ced-3*. These nonsense *ced-3* mutants confirmed that the *ced-3* gene is not essential for viability. One of the 12 mutations is an alteration of a conserved splicing acceptor G, and another has a change of a 70% conserved C at the splice site, which could also generate a stop codon even if the splicing is correct. Interestingly, these EMS-induced mutations are in either the N-terminal quarter or C-terminal half of the protein. In fact, 9 of the 12 mutations occur within the region of *ced-3* that encodes the last 100 amino acids of the protein. Mutations are notably absent from the middle part of the *ced-3* gene (Figure 4A).

Ced-3 Protein Contains A Region Rich in Serines

The Ced-3 protein is very hydrophilic and no significantly hydrophobic region can be found that might be a trans-membrane domain (Figure 5). The Ced-3 protein is rich in serine. From amino acid 78 to amino acid 205 of the Ced-3 protein, 34 out of 127 amino acids are serine. Serine is often the target of serine/threonine protein kinases (Edelman, *Ann. Rev. Biochem. 56*:567-613 (1987)). For example, protein kinase C can phosphorylate serines when they are flanked on their amino and carboxyl sides by basic residues (Edelman, 1987 *supra*). Four of the serines in the Ced-3 protein are flanked by arginines (Figure 6A). The same serine residues might also be the target of related Ser/Thr kinases.

To identify the functionally important regions of the Ced-3 protein, genomic DNAs containing the *ced-3* genes from two related nematode species, *C. briggsae* (SEQ ID NO: 5) and *C. vulgaris* (SEQ ID NO: 6) were cloned and sequenced. Sequence comparison of the three *ced-3* gene products showed that the non-serine-rich region of the proteins is highly conserved (Figure 7). In *C. briggsae* and *C. vulgaris*, many amino acids in the serine-rich region are dissimilar compared to the *C. elegans* Ced-3 protein. It seems that what is important in the serine-rich region is the overall serine-rich feature rather than the exact amino acid sequence.

This hypothesis is also supported by analysis of *ced-3* mutations in *C. elegans*: none of the 12 EMS-induced mutations is in the serine-rich region, suggesting that mutations in this region might not affect the function of the Ced-3 protein and thus, could not be isolated in the screen for *ced-3* mutants.

EXAMPLE 2

A COMMON MECHANISM OF CELL DEATH IN VERTEBRATES AND INVERTEBRATES

Results from previous studies reported in the scientific literature suggest that cell deaths in a variety of organisms, including vertebrates as well as invertebrates, share a common mechanism which involves the activation of genes. These studies are consistent with the hypothesis that genes similar to the *C. elegans ced-3* and *ced-4* genes may be involved in the cell deaths that occur in vertebrates, although certain observations have led some to distinguish vertebrate cell deaths from the programmed cell deaths observed in such invertebrates as

nematodes and insects. Some vertebrate cell deaths share certain characteristics with the programmed cell deaths in *C. elegans* that are controlled by *ced-3* and *ced-4*. For example, up to 14% of the neurons in the chick dorsal root ganglia die immediately after their births, before any signs of differentiation (Carr and Simpson, *Dev. Brain Res. 2*:57-162 (1982)). Genes like *ced-3* and *ced-4* could well function in this class of vertebrate cell death.

Genetic mosaic analysis has suggested that *ced-3* and *ced-4* genes are expressed by cells that undergo programmed cell death, so that these genes may not act through cell-cell interactions (Yuan and Horvitz, *Dev. Biol. 138*:33-41 (1990)). Many cell deaths in vertebrates seem different in that they appear to be controlled by interactions with target tissues. For example, it is thought that a deprivation of target-derived growth factors is responsible for vertebrate neuronal cell deaths (Hamburger and Oppenheim, *Neurosci. Comment. 1*:39-55 (1982)); Thoenen *et al.*, in: *Selective Neuronal Death*, Wiley, New York, 1987, Vol. 126, pp. 82-85). However, even this class of cell death could involve genes like *ced-3* and *ced-4*, since pathways of cell death involving similar genes and mechanisms might be triggered in a variety of ways. Supporting this idea are several *in vitro* and *in vivo* studies which show that the deaths of vertebrate as well as invertebrate cells can be prevented by inhibitors of RNA and protein synthesis, suggesting that activation of genes are required for these cell deaths (Martin *et al.*, *J. Cell Biol. 106*:829-844 (1988); Cohen and Duke, *J. Immunol. 132*:38-42 (1984); Oppenheim and Prevette, *Neurosci. Abstr. 14*:368 (1988); Stanisic *et al.*, *Invest. Urol. 16*:19-22 (1978); Oppenheim *et al.*, *Dev. Biol. 138*:104-113 (1990); Fahrbach and Truman, in: *Selective Neuronal Death, Ciba Foundation Symposium*, 1987, No. 126, pp. 65-81). It is possible that the genes induced in these dying vertebrate and invertebrate

cells are cell death genes which are structurally related to the *C. elegans ced-3* or *ced-4* genes.

Also supporting the hypothesis that cell death in *C. elegans* is mechanistically similar to cell death in vertebrates is the observaiton that the protein product of the *C. elegans* gene *ced-9* is similar in sequence to the human protein Bcl-2. *ced-9* has been shown to prevent cells from undergoing programmed cell death during nematode development by antagonizing the activities of *ced-3* and *ced-4* (Hengartner, *et al.*, *Nature 356*:494-499 (1992)). The *bcl-2* gene has also been implicated in protecting cells against cell death. It seems likely that the genes and proteins with which *ced-9* and *bcl-2* interact are similar as well.

EXAMPLE 3

NEW FORMS OF THE CELL DEATH PROTEINS CED-3 AND CED-4 CAN PREVENT PROGRAMMED CELL DEATH IN *C. ELEGANS*

A *ced-3* cDNA encoding a Cys358 to Ala substitution at the active site cysteine can prevent normally occurring programmed cell death in *C. elegans* when overexpressed using a heat shock promoter. The construct used to transform the *C. elegans* strains is shown in Fig. 10.

Representative data as shown in Table 5 demonstrate protective effect which alterations in the active site cysteine confer.

Table 5

Construct	# extra cells in anterior pharynx	Number of animals observed	Range of extra cell counts

none		0.13	40	
	0-1			
HSP-ced-3(C360A) line 1		2.9	8	0-8
HSP-ced-3(C360A) line 2		4.9	9	0-9
HSP-ced-3(C360A) line 3		2.6	9	1-9

Different lines represent independent strains carrying an extrachromosomal array containing the fusion construct and heat shocked at 33°C for 1 hour.

EXAMPLE 4

Peptide Inhibitors of the Interleukin-1β Converting Enzyme (ICE) Arrest Programmed Cell Death of Motoneurons *In Vitro* and *In Vivo*

Programmed cell death (PCD) has been well documented in the lumbar spinal motoneurons of the chick, where approximately 50% of the neurons produced during embryogenesis die before birth (Hamburger, *Am. J. Anat. 102*:365-410 (1958), Hollday and Hamburger, *J. Comp. Neurol. 170*:311-310 (1976), and Oppenheim *et al., J. Comp. Neurol. 177*:87-112 (1978)). Survival of motoneurons is dependent on their interaction with muscle targets, since removal of the limb induces greater than 90% motoneuron death whereas transplantation of a supernumerary limb increases the number of surviving motoneurons (Hamburger, *Am J. Anat. 102*:365-410 (1958), Hamburger and Oppenheim, *Neurosci. Comm. 1*:39-55 (1982), and Hollday and Hamburger, *J. Comp. Neurol. 170*:311-320 (1976)). While a precise factor has yet to be identified, the supply of target-derived trophic support is critical in determining the extent of motoneuron survival. The death of motoneurons that fail to acquire adequate supply of support

appears to be mediated by new gene expression (Oppenheim *et al., Dev. Biol. 138*:104-113 (1990) and Milligan *et al., J. Neurobiology 25*:1005-1016 (1994)).

The aspartate-directed substrate specificity of ICE has allowed for the development of peptide inhibitors that are potent inhibitors of ICE proteolytic activity (Thornberry *et al., Nature 356*:768-774 (1992)). Those compounds mimic the aspartic acid in the P1 position of known ICE substrates, and are thus active site inhibitors. As such, these compounds may also be expected to inhibit other ICE family members that retain asp-ase activity. In this example ICE inhibitors were used to demonstrate the role of ICE-like proteases in the death of chick spinal motoneurons and demonstrate that cell-permeable peptide inhibitors of ICE arrest the PCD of motoneurons *in vitro* and *in vivo*. Furthermore, these inhibitors can also **reduce** PCD in other cell lineages *in vivo*.

A tissue culture model system that allows isolation of a relatively pure population of motoneurons whose survival is dependent on muscle extract, a potent source of target-derived trophic support, was used to test the ability of peptide inhibitors of ICE to block motoneuron cell death (Milligan *et al., J. Neurobiology 25*:1005-1016 (1994) and Block-Gallego *et al., Development. 111*:221-232 (1991)). The death of motoneurons deprived of trophic support *in vitro* requires new gene expression and occurs by apoptosis (Milligan *et al., J. Neurobiology 25*:1005-1016 (1994)). Motoneurons deprived of trophic support at the time of plating become irreversibly committed to undergo cell death after 16-18 hours (Milligan *et al., J. Neurobiology 25*:1005-1016 (1994)). Figs. 11A and 11B show that treatment with peptide inhibitors of ICE during this time period at concentrations known to be effective in blocking IL-1 maturation in intact cells (Thornberry *et al., Nature 356*:768-774 (1992)), substantially prevents the motoneuron death observed after 3 days. Administration of either a reversible

peptide aldehyde (Acetyl-Tyr-Val-Ala-Asp-addehyde)-(Acetyl-Tyr-Val-Ala-Asp-chloromethyl-ketone) or an irreversible peptide chloro-methylketone (Acetyl-Tyr-Val-Ala-Asp-aldehyde)-(Acetyl-Tyr-Val-Ala-Asp-chloro-methylketone) protease inhibitor had inhibitory effects on motoneuron death, although the peptide aldehyde was more effective. Treatment with the ICE inhibitors had no affect on cells receiving muscle extract, indicating that they are not toxic to motoneurons at the doses tested (Figs. 11A - 11B). Treatment with control peptide aldehyde or cholоromethylketone inhibitors that lack aspartate in the P1 position had no survival promoting effects, further suggesting that it is the specific inhibition of ICE or ICE-like asp-ases that inhibit death (Figs. 11C-11E). When motoneurons are treated with peptide inhibitors in the absence of muscle extract for three days and subsequently supplemented on day three with muscle extract, they continue to survive, and by six days appear as healthy and differentiated as the motoneurons that were continuously supplied with muscle extract (Figs. 12A, 12B, 15G). Thus, motoneurons rescued for 3 days by ICE inhibitors remain capable of responding to trophic factors present in muscle extract. These results suggest that the commitment to cell death initiated by trophic factor deprivation *in vitro* involves an ICE-like asp-ase.

To demonstrate the physiological relevance of the observations obtained *in vitro* the role of ICE-like proteases on several models of PCD in the chick embryos *in vivo* was investigated. In the first model, embryos were treated with a single dose of an ICE inhibitor or control protease inhibitor on embryonic day 8 (E8), the time of maximum naturally occurring motoneuron cell death (Hamburger *et al., J. Morph. 88*:49-92 (1951)). Embryos treated *in vivo* with the peptide inhibitors appeared to develop normally and there were no gross abnormalties. However, 15 hours following drug treatment, there are significantly fewer

pyknotic cells present in the lumbar spinal cord of animals treated with the peptide inhibitors of ICE as compared to animals treated with control protease inhibitors (Table 6A). This effect is dose dependent (Fig. 13). 24 hours after treatment there is a similar reduction in the number of pyknotic cells and a significant increase in the number of healthy motoneurons (Table 6B). The increase in healthy cells suggests that cell death was indeed inhibited and that there was not simply a morphological change in the dying neurons that precluded their identification as pyknotic. The ability of ICE inhibitors to block the naturally occurring cell death of interdigital cells in the developing limb was also studied. These cells undergo PCD as a means of sculpting the digits in many vertebrates. When embryos were treated with either ICE inhibitor on embryonic days, E6 and E7, there was a substantial reduction in the number of pyknotic cells between the digits of the hindlimb (Fig. 14). These data support the theory that ICE-like asp-ases are key components of the PCD pathway in multiple cell types, including neurons and non-neurons.

Next, a second model of motoneuron PCD in which animals were subjected to limb bud removal, thereby inducing greater than 90% of the motoneurons to die was investigated (Hamburger, *Am. J. Anat. 102*:365-410 (1958), Hamburger *et al., Neurosci. Comm. 1*:39-55 (1982). Surprisingly in this model peptide inhibitors of ICE had no survival promoting effect (Table 7).

The ability of the ICE inhibitors to block cell death in two classes of neurons whose naturally occurring death appears to be independent of target interaction was also studied. First, motoneurons in the cervical spinal cord undergo PCD between E4 and E5, with maximum levels occurring at E4.5. It is believed that these cells die by a means independent of target interactions since a variety of growth factors shown to be effective in rescuing lumbar motoneurons

are ineffective in rescuing cervical motoneurons from PCD. Peptide inhibitors of ICE have no survival promoting effects on cervical motoneurons *in vivo* (Table 8). Second, the death of undifferentiated neurons and precursor cells that occurs in the neural tube between E2 and E3 is also thought to be target independent and unaffected by treatment with a variety of growth factors (Homma *et al., J. Comp. Neurol. 345*:377-95 (1994)). The PCD of these cells is also insensitive to rescue by the peptide inhibitors (Table 9). Although one cannot exclude the possibility that the dosage or timing of peptide administrations account for the observed lack of inhibition, this is unlikely given the positive effects on interdigital cell death observed at a similar embryonic stage (see above). These results show that not all naturally occurring cell deaths are blocked by inhibitors of ICE or related aspartases.

While we do not wish to bind ourselves to a particular model, several hypotheses can be offered to explain the differential ability of ICE inhibitors to block motoneuron death in the various models. It is possible, for instance, that the peptides are not present at critical PCD commitment times in each model. In the limb bud extrepation model, where the limb bud is removed at E2, perhaps an asp-ase-sensitive step occurred prior to E5 when drugs were applied. Further time course experiments will clarify this issue. A second possibility is that the molecular machinery that controls cell death changes during the course of development. The two cases where the ICE inhibitor blocked PCD were at E8 (motoneuron death) and E7.5 (interdigital cell death). The cases in which the peptides were ineffective were at E6 (limb bud extrepation), E4 (cervical spinal motoneurons) and E3 (undifferentiated neurons and precursors). Thus it is possible that early in development, cell death mechanisms occur which may not be

inhibited by asp-ase inhibitors employed in these studies, while at later times, different mechanisms are inhibited by the compounds employed.

The experiments described in this example demonstrate that ICE or an ICE-like asp-ase have a regulatory role in vertebrate cell death *in vivo*. The inhibitors used in the present example were designed as asp-ase inhibitors, and inhibit not only ICE, but also related proteases that have been implicated in apoptosis and that resemble ICE with respect to cleavage after apportate residues. The effect of cell-permeable inhibitors on the ICE protease family may result in the arrest of motoneuron death. Such arrest may subsequently allow time for the cell to reorganize and recover, thereby opening up therapeutic strategies in pathological conditions involving motoneuron death such as that which occurs following spinal cord injuries or stroke. In addition, motoneuron death as observed in neurodegenerative diseases (e.g., ALS) might also be prevented by treatment with these asp-ase inhibitors.

Methods

Methods for motoneuron cultures. Spinal cords from embryonic day 5 chicks were dissected in cold phosphate buffered saline (pH 7.4; PBS), incubated in trypsin (0.25% in PBS; Gibco) and the tissue dissociated by passing it several times through a 1.0 ml pipette tip. Cells were layered onto a 6.8% metrizamide (Serva) cushion, centrifuged at 500g. The cell layer at the interface, containing predominantly motoneurons, was collected. Motoneurons were plated onto 12 mm glass coverslips (Fisher) that were initially coated with poly-ornithine (1 μg/ml; Sigma), washed extensively with dH_2O and subsequently coated with laminin (20ug/ml; Gibco). A culture medium containing Leibovitz's L15 media (Gibco) supplemented with sodium bicarbonate (625 μg/ml), glucose (20 mM), progesterone (2×10^{-8} M; Sigma), sodium sclenite (3×10^{-8} M; Sigma),

conalbumin (0.1 mg/ml; Sigma), putrescine (10^{-4} M; Sigma), insulin (5 µg/ml; Sigma) and penicillin-streptomycin (Gibco) was used. Unless otherwise noted, 1 ml of complete media, with or without muscle extract (MEX) (20 µg/ml; prepared as previously described; Bloch-Gallego et al., *Development* 111:221-232 (1991)), was added to the tissue culture wells that contained a coverslip seeded with cells (1 X 10^4 cells/coverslip). We have previously shown that motoneurons in culture become committed to die approximately 16 hours after culture in the absence of MEX (Oppenheim et al., *Dev. Biol.* 138:104-113 (1990)). For these experiments, motoneurons were treated with control protease inhibitors or with peptide inhibitors of ICE (see "peptides" below). The calpain inhibitor Ed64 was purchased from Sigma (St. Louis, MO).) motoneurons were treated with the protease inhibitors every two hours between 14 and 24 hours in culture (the time when cells in the absence of MEX are dying). Treatment with the peptide inhibitor was accomplished by adding the appropriate concentration of peptide to the cells so that the final concentration in the well after the final application would be as indicated in the figures; no more than 0.5% of the total volume of media was added at any time. Aldehyde peptide inhibitors were diluted in dH_2O and chloromethylketone inhibitors were diluted in DMSO. After a total of three days in culture, cells were incubated with the monoclonal antibody SCl (1:5 of supernatant in PBS; 15) for 1.5 hours at 37°C, washed with PBS, fixed with 10% formaldehyde in PBS and subsequently incubated with an FITC-labeled goat anti-mouse IgG secondary antibody (1:50 diluted in PBS; Fisher). After extensive washes with PBS, the cells were incubated with the fluorescent DNA intercalating dye, 4',6-Diamidino-2-phenylindole (DAPI; 1:100,000 in PBS; Sigma) and mounted with the aqueous mounting media Gel-Mount (Biomeda). Surviving motoneurons were counted in 5 predetermined 40X objective fields. For a

motoneuron to be considered viable, its cell body must be present in the filed of view, exhibit uniform SC1 immunoreactivity on its surface membrane and possess a uniform, non-condensed DAPI stained nucleus.

Peptides. Two peptide inhibitors of ICE, Acetyl-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO) and Acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK) and two control peptide inhibitors, Acetyl-Leu-Leu-Arg-aldehyde (Ac-LLR-CHO; Leupeptin) and n-tosyl-Lys-chloromethyl-ketone (Tos-Lys-CMK) were synthesized by Bachem Biosciences (King of Prussia, PA) and shown by thin layer chromatography and HPLC to be greater than 98% pure. Ac-YVAD-CHO is a reversible, competitive inhibitor of human ICE and has been shown to inhibit ICE activity in intact monocytic cells (Thornberry et al., *Nature* 356:768-774 (1992)).

Methods for administration of peptides to examine effects on naturally occurring motoneuron cell death *in vivo*. Although cell death of lumbar motoneurons occurs between E6 and E12, the peak period of death (i.e., the greatest number of pyknotic cells) occurs on E8 (Oppenheim et al., *J. Comp. Neurol.* 177:87-112 (1978)). For these experiments, embryos were given a single administration of an agent on E8 0 hr and sacrificed 15 hours later. 400 µg was chosen since this was the most effective dose tested (Fig. 13). Ac-YVAD-CMK and Tos-Lys-CMK were administered in a solution of DMSO/BSA, whereas Ac-YVAD-CHO and leupeptin (Sigma) were in BSA alone. The solutions (50-100 µl) were dropped onto the highly vascularized chorioallantoic membrane through a window in the shell. The control groups included both DMSO and BSA or vehicle alone. Embryos were killed and staged by the Hamburger-Hamilton series

(Hamburger and Hamilton, *J. Morph.* 88:49-92 (1951)). The thoraco-lumbar spinal cord was dissected, fixed in Carnoys or Bouins fixative, processed for paraffin histology, serially sectioned (10-12 μm) and stained with either thionin or hematoxylin and eosin. Pyknotic motoneurons were identified based on criteria previously described (Chu-Wang and Oppenheim, *J. Comp. Neurol.* 177:33-58 (1978); Clarke and Oppenheim In *Methods in Cell Biology Series; Cell Death*; eds: Schwartz and Osborne. Academic Press. New York, NY. In press.) and were counted in every 10th or 20th section through the entire lumbar enlargement. The total number of pyknotic cells were then estimated by multiplying these values by 10 or 20. All cell counts were performed by individuals blinded with regard to drug treatment of the embryos.

Methods for limb bud removal experiments. A unilateral limb bud removal was performed on E2 as described previously (Oppenheim et al., *J. Comp. Neurol.* 177:87-112 (1978)). Because induced cell death following limb removal begins on E5 (before that there is no difference in the number of motoneurons between the operated or unoperated sides) embryos were given one treatment of Ac-YVAD-CHO (40 μg) or BSA (control) at E5-0 hr and another at E5-12 hours (total peptide administered was 80 μg). Animals were killed at E6-0 hr. Methods were the same as described above except the section thickness was 6-8 μm (see Method for Administration of Peptides, above).

Methods for limb bud interdigital regions. Embryos were treated with 100 μg of peptide or vehicle in 50 μl on E6.0 and on E7.0 (total 200 μg) as described above (Chu-Wang and Oppenheim, *J. Comp. Neurol.* 177:33-58 (1978); Clarke and Oppenheim In *Methods in Cell Biology Series; Cell Death*; eds: Schwartz and

Osborne. Academic Press. New York, NY. In press.) and killed at E7.5. The footpads were placed in Bouin's fixative and processed as described above (see Method of Limb Bud Removal, above). Pyknotic cells in all interdigital regions were counted in every 10th section (6-8 μm) of serial transverse sections through the entire footpad.

Table 1

Sites of Mutations in the *ced-3* Gene

Allele	Mutation	Nucleotide	Codon	Consequence
n1040	C to T	2310	27	L to F
n718	G to A	2487	65	G to R
n2433	G to A	5757	360	G to S
n1164	C to T	5940	403	Q to termination
n717	G to A	6297	-	Splice acceptor loss
n1949	C to T	6322	412	Q to termination
n1286	G to A	6342	428	W to termination
n1129	C to T	6434	449	A to V
n1165	C to T	6434	449	A to V
n2430	C to T	6485	466	A to V
n2426	G to A	6535	483	E to K
n1163	C to T	7020	486	S to F

Nucleotide and codon positions correspond to the numbering in Figure 3.

Table 2

***ced-3-lacZ* Fusions Which**

Prevent Programmed Cell Death

Strain Name	Construct	Average # Extra Cells	Number of Animals
N2 (wild-type)	-	0.1	40
nEx 121	PBA	2.0	23
nEx 70	PBA	2.4	31
nEx 67	BGAFQ	2.1	18
nEx 66	BGAFQ	2.1	25

Table 3

Summary of Transformation Experiments

Using Cosmids in the *ced-3* Region

Cosmid injected	No. of non-Unc transformants	Ced-3 phenotype	Strain name
C43C9; C14G10	1	-	MT4302
W07H6; C14G10	3	-	MT4299
		-	MT4300
		-	MT4301
C48D1; C14G10	2	+	MT4298
		+	MT4303

Animals injected were of genotype: *ced-1(e1735)*; *unc-31(e929) ced-3(n717)*.

Table 4

The expression of *ced-3(+)* transformants

Genotype	DNA injected	Average No. cell deaths in L1 head	No. Animals scored
ced-1	-	23	20
ced-1; ced-3	-	0.3	10
ced-1; nIS1 unc-31 ced-3	C48D1; C14G10	16.4	20
ced-1; unc-31 ced-3; nIS1/+		14.5	20
ced-1; unc-31 ced-3; nEX2	C48D1; C14G10	13.2	10/14
		0	4/14
ced-1; unc-31 ced-3; nEX10	C48D1-28; C14G10	12	9/10
		0	1 of 10
ced-1; unc-31 ced-3; nEX9	C48D1-28; C14G10	12	10
ced-1; unc-31 ced-3; nEX11	C48D1-43 C14G10	16.7	10/13
		Abnormal cell deaths	3/13

Table 4 continued

ced-1; unc-31 *ced-3; nEX13*	pJ40; C14G10	13.75	4/4
ced-1; unc-31 *ced-3; nEX17*	pJ107de128, pJ107de134 C14G10	23	12/14
		0	2/14
ced-1; unc-31 *ced-3; nEX18*	pJ107de128, pJ107del134 C14G10	12.8	9/10
		0	1/10
ced-1; unc-31 *ced-3; nEX19*	pJ107de128, pJ107de134 G14G10	10.6	5/6
		0	1/6
ced-1; unc-31 *ced-3; nEX16*	pJ107del12, pJ107del27 C14G10	7.8	12/12

Alleles of the genes used are *ced-1(e1735)*, *unc-31(e928)*, and *ced-3(n717)*.

Table 6A

Pyknotic Lumbar Motoneurons on E8

(15 hours post treatment)

Control	ICE Inhibitors		Control Protease Inhibitors	
	Ac-YVAD-CHO	Ac-YVAD-CMK	Leupeptin	Tos-Lys-CMK
286±37 (20)	146±35* (8)	216±27** (10)	310±40 (20)	285±31 (20)

*p≤0.001; **p≤0.01

Embryos were treated as described and results are expressed as mean ± SD. Multiple t-tests were performed with the Bonferroni correction. P-values were the same for comparisons of Ac-YVAD-CHO or Ac-YVAD-CMK with control, Ac-LLR-CHO or Tos-Lys-CMK treated animals. The number in brackets represents the n for each group.

Table 6B

Pyknotic and Healthy Lumbar Motoneurons on E9

(24 hours post treatment)

Control	ICE Inhibitors		Control Protease Inhibitors	
	Ac-YVAD-CHO	Ac-YVAD-CMK	Leupeptin	Tos-Lys-CMK
Pyknotic Motoneurons				
316±47 (21)	150±30** (6)	200±33** (6)	297±51 (20)	345±39 (22)
Healthy Motoneurons				
13,605±890 (20)	15,680±684** (6)	16,231±755* (7)	14,117±971 (18)	13,257±773 (19)

*p≤0.001; **p≤0.01

Embryos were treated as described (17) except that they were killed on E9-0 hr (24 hours after treatment), results are expressed as mean ± SD. Multiple t-tests were performed with the Bonferroni correction. P-values were the same for comparisons of Ac-YVAD-CHO or Ac-YVAD-CMK with control, Ac-LLR-CHO or Tos-Lys-CMK treated animals. The number in brackets represents the n for each group.

Table 7

Pyknotic and Healthy Lumbar Motoneurons on E6 Following Limb Bud Removal.

	Ipsilateral		Contralateral	
	Control	Ac-YVAD-CHO	Control	Ac-YVAD-CHO
Pyknotic Motoneurons	375±74* (6)	317±89* (6)	73±16 (6)	67±13 (6)
Healthy Motoneurons	11,371±1780* (6)	11,109±1592* (6)	18,455±1661 (6)	17,914±1733 (6)

*$p \leq 0.001$ Ipsilateral vs Contralateral

Embryos were treated as described and results are expressed as mean ± SD. Multiple t-tests were performed with the Bonferroni correction. The aldehyde peptide inhibitor of ICE, Ac-YVAD-CHO had no survival promoting effect as compared to the unoperated contralateral side. The number in brackets represents the n for each group.

Table 8
Pyknotic Cervical Motoneurons on E4.5.

Control	ICE Inhibitors		Control Protease Inhibitor
	Ac-YVAD-CHO	Ac-YVAD-CMK	Leupeptin
24.1±3.1 (7)	25.9±3.4 (6)	26.7±2.2 (6)	32.5±7.9 (3)

Embryos were treated as described and results are expressed as mean ± SD. Multiple t-tests were performed with the Bonferroni correction. The peptide inhibitors of ICE, Ac-YVAD-CHO or Ac-YVAD-CMK had no survival promoting effects on cervical motoneurons as compared to control or Ac-LLR-CHO treated animals. The number in brackets represents the n for each group.

Table 9. Pyknotic Cells in the Early Neural Tube.

Floor Plate			Dorsal Spinal Cord		
Control	ICE Inhibitors		Control	ICE Inhibitors	
	Ac-YVAD-CHO	Ac-YVAD-CMK		Ac-YVAD-CHO	Ac-YVAD-CMK
0.898	1.115	0.872	1.322	1.350	1.372
±0.103	±0.246	±0.201	±0.471	±0.297	±0.235
(6)	(6)	(5)	(6)	(6)	(5)

Embryos were treated as described and results are expressed as mean ± SD. Multiple t-tests were performed with the bonferroni correction. The peptide inhibitors of ICE, Ac-YVAD-CHO or Ac-YVAD-CMK, had no survival promoting effects on floorplate or dorsal spinal cord cells as compared to control animals. The number in brackets represents the n for each group.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. For example, functional equivalents of DNAs and RNAs may be nucleic acid sequences which, through the degeneracy of the genetic code, encode the same proteins as those specifically claimed. Functional equivalents of proteins may be substituted or modified amino acid sequences, wherein the substitution or modification does not change the activity or function of the protein. A "silent" amino acid substitution, such that a chemically similar amino acid (e.g., an acidic amino acid with another acidic amino acid) is substituted, is an example of how a functional equivalent of a protein can be produced. Functional equivalents of nucleic acids or proteins may also be produced by deletion of nonessential sequences.